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Study of O-GlcNAc modification during capacitation of human sperm

Stamatiadis, Panagiotis

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Panagiotis Stamatiadis

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Study of O-GlcNAc modification during capacitation of human sperm

Panagiotis Stamatiadis

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Declaration

All data was gathered by me alone, and I am the sole author of the text. I have personally consulted all references and I have not submitted this thesis previously for any other degree.

Panagiotis Stamatiadis

Project Summary

Capacitation is the process that renders spermatozoa competent for fertilization. Part of the process of capacitation is hyperactivation. Hyperactivation is a type of motility acquired by sperm in the female reproductive tract and its main characteristics are high flagellar bend amplitude and asymmetrical beating of the tail. The resulting swimming style, which is erratic and often non-progressive, is required for the infiltration of the cumulus oophorus, the penetration of zona pellucida and successful *in vivo* fertilization. Several clinical studies in human suggest that inhibition of hyperactivated motility is linked to male infertility *in vivo* and *in vitro*. The whole induction of hyperactivation is calcium dependent and is accompanied by a cascade of biochemical and physiological changes. Protein phosphorylation represents a very important aspect of hyperactivation, as it is one of the cell's regulatory mechanisms to control various processes. Addition or removal of phosphate groups from serine, threonine or tyrosine residues is one of the most common mechanisms for regulating protein activity. In this project, for the first time we report the discovery of O-GlcNAc and O-GlcNAcylation as a novel form of post-translational modification in human sperm. Furthermore by using Computer Assisted Semen Analysis (CASA) for the monitoring of sperm hyperactivation, along with highly potent inhibitors of O-GlcNAcylation, I discovered impaired hyperactivation, after induction of O-GlcNAcylation during capacitation of human sperm. All of the motility experiments were followed by protein analysis, which revealed that a time-dependent decrease in O-GlcNAcylation occurs during capacitation. Since mature spermatozoa are highly compartmentalized, terminally differentiated and specialized cells, post-translational modifications play a huge role in the regulation of protein function. The discovery of a novel modification in human sperm, which affects hyperactivation, will give us mechanistic insights of the control of hyperactivation.

List Of Abbreviations

AC: Adenylyl cyclase

ALH: Amplitude of lateral head displacement

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CASA: Computer-assisted sperm analysis/analyzer

CM: Capacitating medium

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

ICSI: Intra-cytoplasmic sperm injection

IVF: In vitro fertilization

LIN: Linearity

NCB: Non-capacitating buffer

O-GlcNAcylation: O-GlcNAc Post-Translational Modification

PKA: Protein kinase A

STR: Straightness

VAP: Average path velocity

VCL: Curvilinear path velocity

VSL: Straight line velocity

WHO: World Health Organization

Chapter 1: General Introduction

1.1 Spermatogenesis

Spermatogenesis is the process by which male Primordial Germ Cells (PGCs) undergo meiosis and develop to mature spermatozoa (figure 1-1) (Avarbock et al., 1996). PGCs originate from the yolk sac (extra embryonic endoderm) and migrate into the genital ridges (Kierszenbaum, 2002).

1.1.1 Location

Production of the sperm cell takes place within several structures of the male reproductive system. The seminiferous tubules of the testes are the starting point of the process. The cells gradually migrate to the epididymis where the maturation process occurs (de Kretser et al., 1998a). Mature cells (sperm cells) are stored in the epididymis until ejaculation. The whole process for humans takes approximately 64 days (Heller and Clermont, 1963) and is regulated by endocrine and paracrine systems. Histone modification and DNA methylation are implicated in the regulation of this process (Song et al., 2011, Baarends et al., 1999).

1.1.2 Stages

The whole process is comprised of two major steps. The first step is spermatocytogenesis, in which diploid primordial germ cells located in the basal compartment of seminiferous tubules undergo mitosis and give rise to two primary spermatocytes. PGCs continuously renew their population by mitotic replication (de Kretser et al., 1998b). Each primary spermatocyte then moves into the adluminal compartment of the seminiferous tubules and undergoes meiosis to produce two haploid primary spermatocytes, which later on will divide into spermatids. In the second step of the process, spermiogenesis, the spermatids develop and give rise to mature sperm cells. Spermiogenesis is a series of events.

The changes include:

1. Formation of sperm flagella(Inaba, 2011)
2. Polymerization of tubulin and formation of microtubules(Bergen and Borisy, 1980)
3. Chromatin condensation in the nucleus(Brewer et al., 2002)
4. Migration of the nucleus to the periphery of the cell(Foster et al., 2005)
5. Formation of the acrosome(Abou-Haila and Tulsiani, 2000)
6. Phagocytosis of the cytoplasm by the Sertoli cell and formation of the residual body (de Kretser et al., 1998b).

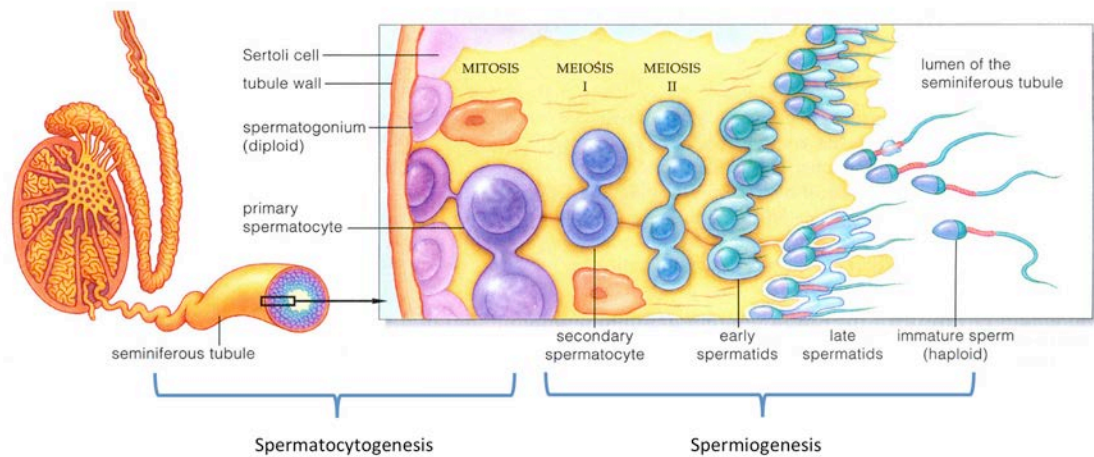


Figure 1-1 The above figure describes the organisation and the basic steps of the process of spermatogenesis. The whole process is comprised of two major steps, spermatocytogenesis and spermiogenesis. Primordial germ cells located in the seminiferous tubules undergo spermatogenesis and give rise to mature sperm cells that are stored in the epididymis (Avarbock et al., 1996).

1.1.3 Regulation

Neuroendocrine cells in the hypothalamus stimulate the pituitary gland by pulsatile secretion of gonadotropin releasing hormone (GnRH). The GnRH after is released into the portal blood system, binds to specific membrane receptors in the gonadotropic cells of the anterior pituitary. The pituitary gland gets stimulated and releases pulses of LH and a continuous stream of FSH to the peripheral blood (Blache et al., 2000). The role of the above two gonadotropins (glycoprotein hormones), FSH and LH are very important in the regulation of the production of spermatozoa in the testis(Leung and

Steele, 1992). They share similar chemical and structural features. Both glyco-hormones are composed of similar α -subunit, but the β -subunit has unique sequence for each hormone. The pulses of LH that are released into the blood stream cause the stimulation of Leydig cells and production of testosterone and other androgens. The testosterone is converted into dihydro-testosterone (DHT) and estrogen, necessary for spermatid maturation.

1.1.4 PTM during spermatogenesis and epididymal maturation

The development of functional spermatozoa is accompanied by carefully orchestrated changes, controlled by independent regulatory mechanisms in distinct subcellular compartments. Tyrosine phosphorylation is one of these changes in protein function that has been recognized as an important component of sperm differentiation. cAMP-induced tyrosine phosphorylation cascade has been studied in depth during the post-ejaculatory events leading up to fertilization but little is known about the role of this pathway during spermatogenesis and epididymal maturation. Tyrosine phosphorylation becomes detectable by the time sperm cells enter the epididymis. Spermatozoa in the cauda epididymis express tyrosine phosphorylated residues initially in the principal piece and subsequently in the midpiece. By the end of these series of maturation events, cells exhibit a cAMP tyrosine phosphorylation response that runs the entire length of the tail from neck to the end-piece. The cAMP-dependent phosphorylation pattern in the presence of extracellular calcium is positively correlated with their capacity to exhibit hyperactivated movement and acquisition of functional competence (Lin et al., 2006).

1.2 Sperm Structure

The male gamete (spermatozoon), is a highly specialized and compartmentalized, nearly cytoplasm-free cell with a very important role (Barratt et al., 2009). It is specifically designed to travel through the cervix of the female reproductive tract, through the uterus and up to the fallopian tube, where it will fertilize the egg. During differentiation

spermatozoon forfeits most of its cytoplasm and organelles to transform into a motile cell capable of travelling in the female reproductive tract. The stripped-down spermatozoon contributes with many ways to the fertilization. It's not only the paternal chromosomes that are being transferred to the egg but also the centrosome, perinuclear material and messenger RNA (mRNA). The spermatozoon is composed of a sperm head and a sperm tail (flagellum) (figure 1-2).

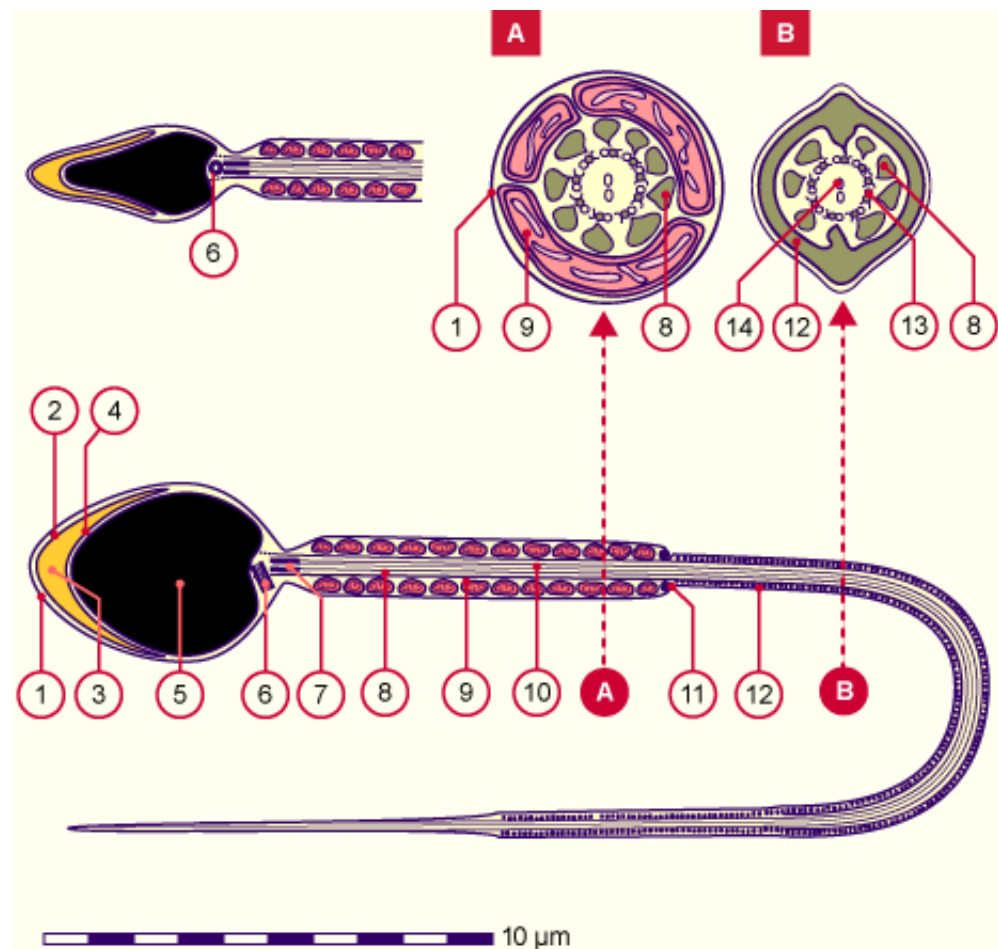


Figure 1-2 The human spermatozoon is spatula shaped and consists of 3 major compartments, a head, a midpiece and a tail. The figure contains a detailed structured analysis of the sperm (Ward and Coffey, 1991).

- 1 Plasma membrane
- 2 Outer acrosomal membrane
- 3 Acrosome
- 4 Inner acrosomal membrane
- 5 Nucleus
- 6 Proximal centriole
- 7 Rest of the distal centriole
- 8 Thick outer longitudinal fibers
- 9 Mitochondrion
- 10 Axoneme
- 11 Anulus
- 12 Ring Fibers
- 13 Peripheral double tubuli
- 14 Central single tubuli

1.2.1 Sperm Head

In humans the head of the sperm is spatula-shaped. It is composed of a nucleus surrounded by cytoplasm. The nucleus is surrounded by a reduced nuclear envelope as the nuclear pore complexes have been removed during spermiogenesis. The reduced envelope covers the haploid genome, which is very condensed and most of the histones (~80%) are replaced by protamines. Protamines are arginine rich proteins essential for sperm head DNA condensation and stabilization (Balhorn, 2007). “Perinuclear matrix” is a structure that covers and offers protection to nucleus. It consists of disulfide bond-stabilized proteins amalgamated with other proteins, which have a bivalent role (Okamoto, 1995, Okamoto and Maravei, 1995). As long as the proteins remain in the complex of the Perinuclear Matrix, their only role is to protect the nucleus. After fertilization occurs, and the proteins are released into the oocyte cytoplasm they function as transcription factors (Sutovsky et al., 2003).

1.2.1.1 Structure of the Perinuclear Matrix:

1. Sub-acrosomal Layer: Functions to anchor the chromosomes
2. Equatorial Segment: It carries several receptor molecules necessary for the initial binding of the sperm to oolemma (egg plasma membrane) (Barratt and Publicover, 2001), which occurs when the spermatozoon reaches the perivitelline space, after the penetration through the cumulus oophorus and the Zona Pellucida (ZP) (Toshimori et al., 1992).
3. Post-acrosomal sheath: Carries several signaling proteins, which are released into the egg cytoplasm, after fusion of the egg with the sperm. The proteins disperse across the ooplasm and trigger signaling pathways responsible for the oocyte activation and initiation of the zygotic development (Sutovsky et al., 2003).

1.2.2 Sperm Flagellum

The sperm tail (flagellum) consists of 4 different segments that share a common inner structure of the microtubules but differ in the external substructure.

1.2.2.1 Inner Structure:

The axoneme of sperm tail consists of a 9+2 arrangement of microtubules. 9 pairs of symmetrically arranged doublets of microtubules connected by dynein arms surround a central pair of microtubules. The microtubules of each doublet are connected each other by nexin links(Bozkurt and Woolley, 1993). The peripheral doublets are connected with the central pair by radial spokes(Smith and Yang, 2004, Goodenough and Heuser, 1985, Witman et al., 1978). Nine outer-dense fibers in parallel position with the outer doublets provide flexible, yet firm support during flagellar movement. The outer-dense fibers consist of serine, proline, and cysteine-rich filament proteins, abundant in zinc-dependent disulfide cross bridging.

1.2.2.2 Segments of the sperm tail (figure 1-3):

- 1) Connecting Piece: The connecting piece, in most mammals (except rodents), contains the sperm proximal centriole inside the dense mass of capitulum. The capitulum is caged inside 9-segmented columns that are a direct continuation of the outer dense fibers in the other flagellar segments.
- 2) Midpiece: Midpiece contains all the organelles, which generate the necessary energy for sperm flagellar motility. Approximately 75-100 mitochondria form the mitochondrialsheath, which covers the midpiece. Sperm mitochondria have unique characteristics. They possess protein isoforms or proteins that are apparently unique and they are not found in the somatic cell mitochondria. Due to high levels of mutagenic oxidative stress encountered by the spermatozoon, all the paternal mitochondria are eliminated by targeted proteolysis inside the fertilized ovum (Sutovsky et al., 2004).

- 3) **Principal piece:** The principal piece makes up $\sim 2/3$ of the length of the sperm flagellum. The fibrous sheath covers it. Fibrous sheath is a unique structure surrounding the outer dense fibers and the axoneme and is composed of two longitudinal columns running parallel to outer dense fibers 3 and 8. Its unique structure defines the shape of flagellar beat, the degree of flagellar flexibility and the plane of flagellar motion. Most of the proteins isolated from the mouse fibrous sheath contain anchoring sites for cAMP-dependent kinases, necessary for the process of sperm capacitation and hyperactivation prior to fertilization. A-kinase anchoring proteins represent in some species more than 50% of the fibrous sheath mass (Eddy et al., 2003).
- 4) **End Piece:** Contains axonemal doublets and the ends of outer dense fibers and fibrous sheath.

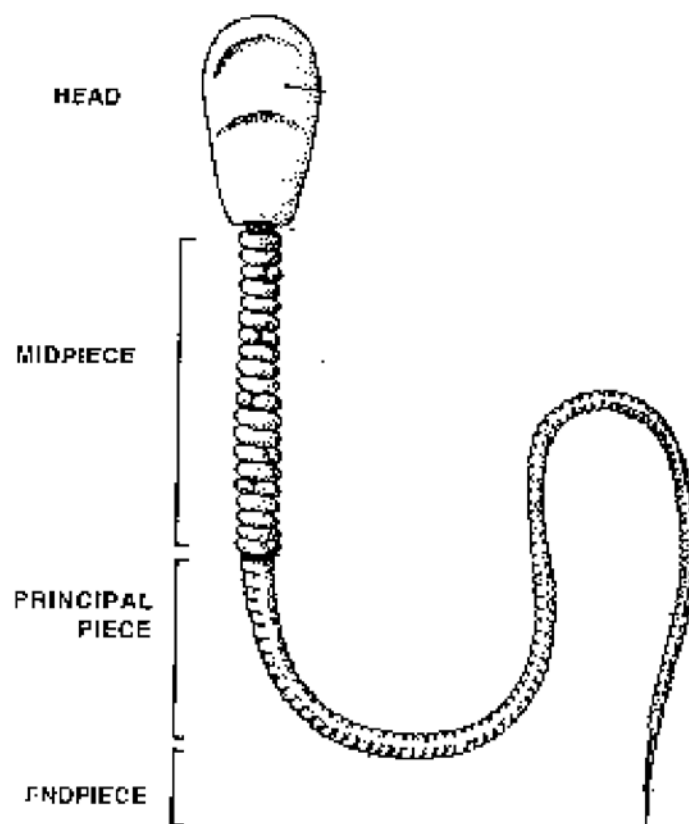


Figure 1-3 The flagellum consists of 4 different segments. The connecting piece located between the head and the midpiece (not shown in the figure), the midpiece, the principal piece and the endpiece (Ward and Coffey, 1991)

1.2.3 Post-translational modifications of microtubules

Microtubules perform multiple diverse functions including, regulation of cell shape, intra-cellular transport and cell motility. Post-translational modifications of tubulin subunits modify the outer and inner surfaces of microtubules providing a mechanism for their functional specialization. Tubulin PTMs that occur on microtubules and have been well characterized include acetylation of lysine residues, de-tyrosination, glycosylation and glutamylation. The use of antibodies that specifically recognize post-translationally modified tubulins reveals differences in the level of PTMs among distinct microtubules, or even along the length of the same microtubule which crucially affects their dynamics and organization (Laurent and Fleury, 1993, Wloga and Gaertig, 2010).

1.2.4 Relationship between functional quality and protein tyrosine phosphorylation

Human semen consists of a heterogeneous cell population with varying degrees of maturation and fertilizing capacity. Protein tyrosine phosphorylation has been associated with certain aspects of sperm physiology such as capacitation, acrosome reaction and hyper-activation (Visconti and Kopf, 1998). Buffone et al. (2004) demonstrated that the subpopulation of sperm isolated between the 45 and 65% of a 45:65:90% Percoll gradient showed a clear impairment capacity to undergo protein tyrosine phosphorylation. Moreover the same population exhibited the lowest percentage of motile and morphologically normal sperm. The reduced capacity to undergo tyrosine phosphorylation could be the result from defects at the plasma membrane originated during spermatogenesis or epididymal maturation. Ollero et al. (2000) reported differences in the sperm membrane lipid composition and particular in the content of docosahexaenoic acid and cholesterol of sperm isolated from a similar gradient. Also work from the same group demonstrated that spermatozoa from varicocele samples (patients with decreased testicular function) presented a decreased response to the capacitating challenge, showing significantly lower motility, hyper-

activation, incidence and intensity of tyrosine phosphorylation, and membrane fluidity (Buffone et al., 2006). The significance of the above lies in the association between structure and functional competence between spermatozoa (Ollero et al., 2000, Buffone et al., 2004).

1.3 Controls Of Sperm Motility

In order to achieve in vivo fertilization, sperm must be able to swim from their point of deposition to the oviductal ampulla (site of fertilization). Sperm's ability to swim is progressively acquired during the epididymal transit. Later on, extrinsic factors secreted by the female genital tract, will trigger capacitation including hyperactivated motility, which is characterized by high flagellar bend amplitude and asymmetrical beating of the tail. Induction of hyperactivated motility is crucial for the sperm to achieve fertilization, because it is required for the penetration of the zona pellucida.

1.3.1 Structure and role of dynein arms

The axoneme is the core structure responsible for motility. Dynein arms, that connect the symmetrically arranged doublets, are responsible for the conversion of chemical energy (ATP) into mechanical energy required for sperm motility. They consist of three classes of subunits:

- 1) Heavy chains: They contain ATPase activity and are responsible for the active sliding across the microtubule
- 2) Intermediate Chains: They are responsible for the anchoring of the arms to the microtubules
- 3) Light Chains: Regulatory function with Calcium binding properties that are required for the fine tuning of the microtubule sliding and the regulation of sperm motility (Inaba, 2002).

The two-dynein arms have separate roles and unique functions. The outer dynein arms are bound to tubulin by the dynein-docking complex and their removal causes the

reduction of the flagellar beat frequency (DiBella and King, 2001, Porter and Sale, 2000, Takada et al., 2002). They produce as much as four/fifths of the force for flagellar movement and it contains three dynein heavy chain proteins, two intermediate and several light chains. The dynein-docking complex (DDC) is necessary for the binding of the outer dynein arm on the axonemal apparatus. On the other hand the inner dynein arms consist of three different classes, equally distributed along the microtubule and they determine flagellar wave form and beating symmetry (Brokaw and Kamiya, 1987).

1.3.2 Dynein Regulatory complex (DRC)

The DRC is a key regulator of motor activity and is bound to the inner dynein arms and functions as a scaffold for enzymes that regulate and modify dynein activity.

Microtubules are cylindrical polymers, made up of polymerized α - and β - tubulin dimers. The dimers are often subjected to post-translational modifications, for example acetylation, palmitoylation, phosphorylation, polyglutamylation and polyglycation, which are important for axonemal motility (Gagnon et al., 1996, Huitorel et al., 2002, MacRae, 1997).

1.3.3 Regulation of dynein arms

Recent genetic and proteomic studies have discovered several new components of axoneme, which are correlated with the generation, and regulation of sperm motility during fertilization. All these components have been well conserved through evolution.

The initiation of movement begins with an activating stimulus, such as changes in ionic conditions or specific substances from eggs or the genital tract, followed by signaling conducted by a second messenger such as cAMP, cGMP or Ca^{2+} which leads to axonemal movement through activation of axonemal dyneins by protein phosphorylation or de-phosphorylation (Morisawa, 1994, Cosson et al., 2008, Chang and Suarez, 2010). The mechanism of dynein activation has not been fully elucidated, but

the phosphorylation of the Tctex-2-related Light Chain (LC) of outer arm dynein and the de-phosphorylation of an Intermediate Chain of inner arm dynein appears to be a prerequisite for the activation of outer or inner arm dynein, respectively (Inaba et al., 1998, Inaba et al., 1999, Nomura et al., 2000, Hozumi et al., 2008).

1.4 Capacitation

Capacitation is a combination of early and late events that renders spermatozoa competent for fertilization. The difference between the early and late events is that the fast processes do not require the addition of cholesterol acceptors such as BSA. Over the years capacitation has been associated with a list of endpoints.

1. Initiation of motility (early event).
2. Increase of cholesterol availability for acceptors (early event).
3. Tyrosine phosphorylation increase (late event).
4. The acquisition of the hyperactivated state, which is considered to be necessary for fertilization (late event).
5. Preparation of the sperm to undergo an agonist induced acrosome reaction (late event).
6. Employment of Chemo taxis (late event) (Eisenbach, 1999).

1.5 Molecular basis of capacitation

Although more than 50 years have passed since the process of capacitation was discovered (Austin, 1951, Chang, 1951) and several pathways have been characterized, the molecular basis of capacitation remains elusive (figure 1-4).

1.5.1 Early Events

Capacitation is a bicarbonate dependent process (Lee and Storey, 1986, Boatman and Robbins, 1991, Shi and Roldan, 1995, Gadella and Harrison, 2000, Visconti et al., 2002). After ejaculation, in the female reproductive tract, the concentration of

bicarbonate in the sperm-surrounding milieu is significantly increased. Most of the bicarbonate enters the sperm through a sodium/bicarbonate co-transporter (Demarco et al., 2003, Romero and Boron, 1999). The movement of the bicarbonate inside the cell induces an increase in intracellular pH (Zeng et al., 1996) and has been associated with the regulation of cAMP metabolism by stimulating soluble Adenylyl Cyclase (sAC) (Bianchini et al., 1990, Garbers et al., 1982). Sperm from sAC deficient mice are not able to capacitate and consequently are infertile (Esposito et al., 2004). Moreover addition of sAC specific inhibitor stops capacitation (early and late events) (Hess et al., 2005). The rise of intracellular cAMP activates Protein Kinase A, which phosphorylates various proteins, causing the initiation of several signaling pathways (Harrison and Miller, 2000, Harrison, 2004) and finally the initiation of sperm motility. In sperm exposed to bicarbonate, cAMP rises to a maximum within 60sec and the observed increase in PKA-dependent phosphorylation begins within 90sec (Salicioni et al., 2007). The pathway is very conserved in spermatozoa and has been identified in all mammalian species (Aitken et al., 1995, Visconti et al., 1995a, Visconti et al., 1995b, Galantino-Homer et al., 1997, Mahony and Gwathmey, 1999, Lewis and Aitken, 2001). PKA catalyzes the addition of the phosphate group to serine and threonine residues. The fact that it's not a tyrosine kinase, suggests that, a protein kinase cascade regulates the process of capacitation. PKA also directly modulates the response of CatSper (voltage gated Calcium channel) to changes in membrane potential (Wennemuth et al., 2003), causes the activation of phospholipids scramblase (Harrison, 1996, Harrison et al., 1996) and increases the availability of cholesterol for external acceptors (Flesch et al., 2001).

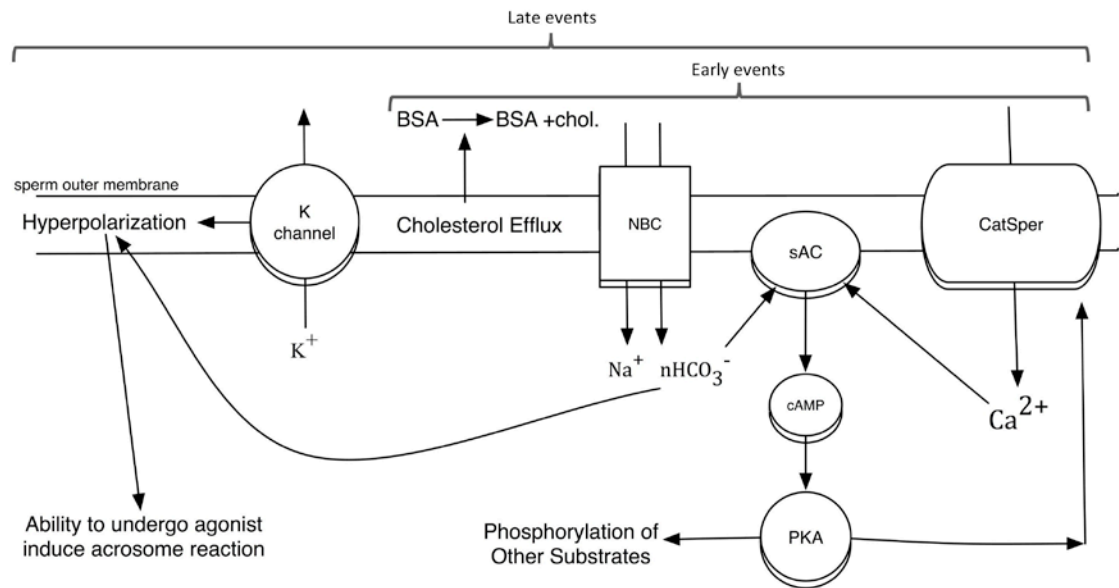


Figure 1-4: The figure contains the biochemical pathways, which regulate early and late events of capacitation. The difference between early and late events is that late events require the presence of cholesterol acceptors

1.5.2 Late Events

Interestingly, both early and late events are centrally regulated by the bicarbonate/sAC/cAMP/PKA pathway, however late events are not instantly stimulated and require the presence of cholesterol acceptors.

The cholesterol acceptors produce an enhanced K⁺ permeability (increased K⁺ efflux), resulting in hyper-polarization of the membrane potential. The mechanism involved is not clear at present. One possibility is that cholesterol efflux redistributes or disrupts lipid rafts, resulting in changes in the activity of ion-selective channels. Hyper-polarization regulates the ability of the sperm to generate transient intracellular Ca²⁺ elevation during an agonist induced acrosome reaction. So the changes in membrane potential are converted into a Ca²⁺ signal. It has been shown that in absence of BSA the changes in membrane potential do not occur and the sperm is unable to undergo an agonist induced acrosome reaction (Demarco et al., 2003).

The report from (Morgan et al., 2008) tries to elucidate the paradox of a centrally regulated pathway, by producing a model that allows the dissection of PKA regulated events in sperm. This Group introduced a point mutation in the ATP binding site of the

PKA catalytic subunit “making room” for the oversized kinase inhibitors that compete ATP for binding. The large substituted inhibitors do not fit in the binding-site of wild-type kinases and therefore would not affect other cellular processes. By incubating with oversized inhibitors they blocked of PKA phosphorylation of substrates, which occurs within 90sec of addition of bicarbonate and as well tyrosine phosphorylation. Blocking of tyrosine phosphorylation was only observed, when mutant sperm was incubated for an extended period of time. This came to the conclusion that PKA during capacitation plays two independent roles in the regulation of sperm motility. A fast role that is required for the activation of flagellar beat and a slow role that is required for the acquisition of the hyperactivated state (Morgan et al., 2008).

Except for PKA, only a few kinases have been described in mature mammalian sperm using antibodies and the identity and functions and their targets in sperm are not well defined. Some of these kinases are:

1. Protein Kinase C (PKC) (Rotem et al., 1990a, Rotem et al., 1990b)
2. GSK3 (Vijayaraghavan et al., 2000)
3. Casein Kinase II (Chaudhry et al., 1991a, Chaudhry et al., 1991b)
4. Testis Specific Serine Kinase (TSSK) (Hao et al., 2004)

Less is known about the tyrosine kinases that mediate the effect of the capacitation-associated increase in protein tyrosine phosphorylation. Some evidence exist for the presence of:

1. C-yes (Leclerc and Goupil, 2002)
2. Src (Baker et al., 2006)
3. ZRK (tyrosine kinase receptor) (Burks et al., 1995)

The presence of csk in mouse sperm is particularly interesting. This kinase negatively regulates src kinase activity and it is at the same time directly inhibited by PKA,

opening the possibility that this pathway is involved in the regulation of the capacitation-associated increase in tyrosine phosphorylation(Baker et al., 2006).

1.6 Journey in the female Oviduct

After ejaculation the spermatozoa have to travel from the point of deposition to the oviductal ampulla, which is the point of fertilization, if ovulation occurs. It is an arduous journey that begins with the passage of the sperm through the ejaculatory ducts(Barratt and Cooke, 1991).The sperm mixes with fluid derived from the seminal vesicles, the prostate and the bulbourethral glands to form the semen(Kvist, 1991). In humans seminal fluid contains several components besides spermatozoa, all necessary for promoting the survival of spermatozoa and provide a nutritive and protective medium in which they can swim.

1.6.1 Uterus and oviductal barriers

In order for the spermatozoa to reach the site of fertilization, oviduct ampulla, they have to pass through many barriers in the uterus and oviduct. Cervical mucus is the first barrier for spermatozoa transportation, which contributes to important functions of sperm cells. The composition of the cervical mucus varies during the menstrual cycle. During the peri-ovulatory period, the mucus being produced by the cervical glands is more hydrate than other times of the menstrual cycle (Morales et al., 1993). Four parameters determine the penetration of spermatozoa through the mucus:

- 1) The motility of spermatozoa
- 2) The number of spermatozoa
- 3) The amount of hydrolytic enzymes present in seminal plasma
- 4) Contractility of the female reproductive tract.

So only sperm cells that move rapidly (hyperactivated), with sufficient velocity in a forward progressive manner will traverse the cervix(Hanson and Overstreet, 1981). Hanson also found that the time from insemination until fertilization to occur can be as

low as 30 minutes (Hanson and Overstreet, 1981). The same study suggests that the mucus present at the uterine-tubal junction or UTJ is rich in glycoproteins, which induce the deprivation of seminal macromolecules from spermatozoa surface. It is also reported that the mucus of the uterine tubal junction flows in the opposite direction to sperm movement, which may represent an additional system by which poor quality sperm is being filtered (Pacey et al., 1995, Kervancioglu et al., 1994, Baillie et al., 1997).

1.6.2 Effect and importance of human cervical mucus and bicarbonate on human sperm motion- hyperactivation

During migration through the mucus, spermatozoa experience complex biophysical, biochemical and morphological interactions affecting their motion (Katz et al., 1989). All these events are of primary importance for their fertilizing capacity. Especially the induction of cAMP dependent tyrosine phosphorylation by bicarbonate, which leads to sperm hyperactivation confers several advantages to spermatozoa, including the development of forces necessary to detach the sperm head from epithelial adherence, the prevention of entrapment within confined spaces and the generation of increased forces to promote the penetration of cumulus and zona (Tesarik et al., 1990, Suarez et al., 1991). Zhu et al. (1992) by examining the effects of exposure to human cervical mucus indicated that mucus promotes hyperactivated motility but spermatozoa had different motility characteristics compared to control samples incubated in capacitating media. The difference maybe is related with a different phosphorylation induced-pattern by the media (mucus or capacitating media).

Although the mechanism by which cervical mucus interacts with spermatozoa still remains unclear, it has been suggested that sperm transport through cervical mucus physically distorts the sperm structure and the physical contact results in macromolecule relocation on sperm proteins attached to plasma membrane (Yudin et al., 1989, Katz et

al., 1989). Here I have to say, that changes in cervical mucus properties and bicarbonate (HCO_3^-) secretion within the female reproductive tract, are critically linked to capacitation but they are 2 different things. Both HCO_3^- and mucus change dramatically throughout the menstrual cycle, but they change independently.

1.6.3 Sperm Chemotaxis

Until now, only two sources of sperm chemo-attractants have been identified. One is the mature oocyte and the other is the cumulus cells surrounding the oocyte (Sun et al., 2005). No chemo-attractants have been identified in the cervical mucus. Progesterone is the main chemo-attractant secreted by the cumulus cells along with atrial natriuretic peptide (ANP) and other specific odorants (Zamir et al., 1993, Spehr et al., 2003). The identity of the chemo-attractants secreted by the oocyte, still remains unknown.

1.7 Fertilization Events

- 1) Passage through the jelly-like matrix of the cumulus oophorus which surrounds the oocyte
- 2) Specific binding to the zona pellucida, a glycoprotein capsule which coats the oocyte
- 3) Exocytosis of acrosomal contents. A process which renders the sperm plasma membrane capable of interacting with the oolemma
- 4) Penetration of the zona pellucida
- 5) Binding and fusion with oolemma.

1.8 Infertility

According to the World Health Organization, infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months of regular unprotected sexual intercourse (Cooper et al., 2010). Conception rates for “normal” fertile couples can be as high as 30% per month and approximately 85% within 1 year

(Spira, 1986, Ford et al., 2000, Thonneau et al., 1991). In every case that the above rates are reduced, we have to account this couple as an “infertile couple”.

Male infertility is a diagnosis of relative impairment in male reproductive potential. Male infertility presents a particularly complex clinical problem. While the patient's semen may seem to be the target for diagnostic and therapeutic interventions and analysis, a positive outcome is in fact manifested by another person, a mother, giving birth to a child. While infertility is relatively common, it is very difficult indeed to establish the relative contribution of the male partner, given the profound difficulties, which exist in the accurate diagnosis of male infertility.

Most studies have used the conventional criteria of semen quality, promulgated by WHO. Although of great importance, these criteria are not evidence-based. They have limited diagnostic value (Irvine and Aitken, 1994) and a significant proportion of men with normal conventional criteria of semen quality will be infertile because of defects in sperm function (Aitken et al., 1982a, Aitken et al., 1991) while a significant number of men with abnormal semen quality will have normal sperm function (Aitken et al., 1982b, Aitken, 1985).

Nevertheless, one common theme to emerge is that, using the available diagnostic techniques; male factor infertility is, in many studies, the commonest single diagnostic category (Collins et al., 1983, Cates et al., 1985, Hull et al., 1985, Haxton and Black, 1987, Randall and Templeton, 1991, Thonneau and Spira, 1991, Schmidt et al., 1995). Contributory male infertility factor is identified in almost 50% of infertile couples while it is the sole cause in 20-30% of infertile couples (Hull et al., 1985, Thonneau et al., 1991).

Despite of the huge importance very little is known about the factors that cause sperm dysfunction. Our limited understanding of these factors disabled us to develop drugs to cure male infertility. Therefore, patients with sperm dysfunction have to rely on

Assisted Reproductive Techniques (ART), which some of them, especially ICSI, have been associate with birth defects. So there is an urgent need in understanding sperm physiology first, and then develop drugs to cure sperm dysfunction or improve the fertilization capacity of the man in order to reduce the usage of ART.

1.9 O-GlcNAcylation

O-GlcNAc is a single sugar (monosaccharide) found on both cytosolic and nuclear proteins. It is attached in a β -linkage (covalent attachment) on serine and threonine residues (hydroxyl-amino acids) during protein post-translational modification. The addition of O-GlcNAc is a reversible procedure.

1.9.1 Historical Background

O-GlcNAcylation was discovered 30 years ago and since then the number of proteins that are O-GlcNAc modified is increasing rapidly. Early studies conducted in viruses and plants, hinted the presence of O-GlcNAc as a linking sugar to hydroxyl-amino acids in proteins. O-GlcNAc was first shown to be a major form of intracellular glycosylation in murine lymphocytes in 1984. These studies used radioactivelylabelled UDP-Gal, which can be used to “tag” on O-GlcNAc residues. 2 years later, in 1986, O-GlcNAc modification was found to be abundant in the cytoplasm and in all subcellular organelles, except mitochondria. Since these early studies, O-GlcNAc has been reported on a large number of proteins. Today, 30 years after the discovery, studies make clear that O-GlcNAc modification is as abundant and dynamic as phosphorylation on many nuclear and cytoplasmic proteins.

Since the sperm is a highly compartmentalized, nearly cytoplasm-free cell, transcriptionally and translationally inactive, the identification of the role of O-GlcNAcylation in sperm and especially capacitation will help us elucidate the molecular pathways, which control the process of capacitation(Hart et al., 1989b, Torres and Hart, 1984b, Kreppel et al., 1997, Hart, 1997, Roquemore et al., 1994).

1.9.2 Subcellular localization

Studies using highly purified Glycosyltransferase and radioactively labelled sugar nucleotides (UDP-Gal) found that O-GlcNAc is abundant in the cytoplasm and in subcellular organelles with higher concentration found in the nuclear envelope (nuclear

pore proteins) and highest number in nucleoplasm. The first studies were conducted with murine lymphocytes 1984 (Torres and Hart, 1984a). In 1986 O-GlcNAc modification was found to be abundant in most of the subcellular organelles of rat liver, except mitochondria (Holt and Hart, 1986). Separate studies also found sites of O-GlcNAc modification on polytene chromosomes of *Drosophila* larvae with markedly increased concentrations at inactive sites (Hart et al., 1989a). This data suggest that the addition of O-GlcNAc not only affects proteins, by altering their function, but also DNA by specific silencing of gene expression. All the proteins that are O-GlcNAc modified can be phosphorylated and may contribute to the formation of multimeric complexes, a process often tightly regulated by phosphorylation (Hu et al., 2010).

1.9.3 Function and Structural analyses of enzymes involved in the O-GlcNAcylation

The dynamic turnover of O-GlcNAcylation occurs on Ser/Thr residues (figure 1-5). Some of these residues can be phosphorylated, but whilst phosphorylation is mediated by over 500 kinases and 140 phosphatases (Manning et al., 2002), only two enzymes are responsible for O-GlcNAc cycling in *Homo sapiens*. These two enzymes are a Glycosyltransferase (OGT) and a glycoside hydrolase (OGA). OGT uses an activated sugar donor to drive the formation of the new glycosidic bond. Particularly OGT takes an α -linked UDP D-sugar to make a β -D glycosidic linkage to the side chain hydroxyls of serine or threonine residues.

On the other hand, OGA takes the D-GlcNAc β -linked to serine/threonine and generates, through hydrolysis, β -D-GlcNAc as product (Martinez-Fleites et al., 2010).

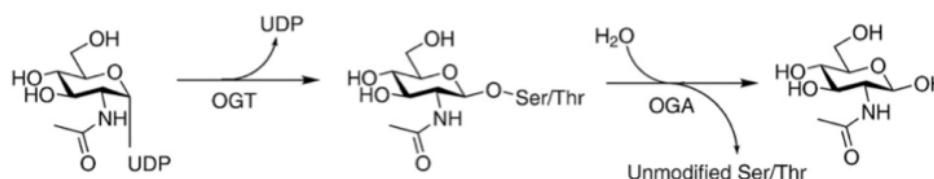


Figure 1-5 The schematic representation of addition and removal of O-GlcNAc. O-GlcNAcylation is described as the addition of a single sugar, O-GlcNAc on serine and threonine residues (Martinez-Fleites et al., 2010).

1.9.3.1 Structural Analysis of OGT

OGT contains two domains, an N-terminal tetratricopeptide repeat (TPR) domain and a C-Terminal catalytic domain, which are separated by a bipartite nuclear localization sequence (NLS). TPR domains produce a super-helical structure containing an asparagine ladder that is critical for protein recognition. Alternative splicing produces multiple isoforms, each with varying numbers of TPRs. This difference allows each isoform to have a different behaviour on a vast array of tissues and on many different protein substrates (figure 1-6). In mammalian organisms OGT appears to be present as

three isoforms. The longest isoform is a nucleo-cytoplasmic OGT (ncOGT) with 12 TPRs. A shorter form of OGT with 9 TPRs and an N-Terminal mitochondrion targeting sequence (mOGT) is mitochondrion specific. Finally an even shorter form of OGT, containing only 2 TPRs (sOGT), is found in the cytoplasm (Hanover et al., 2003).

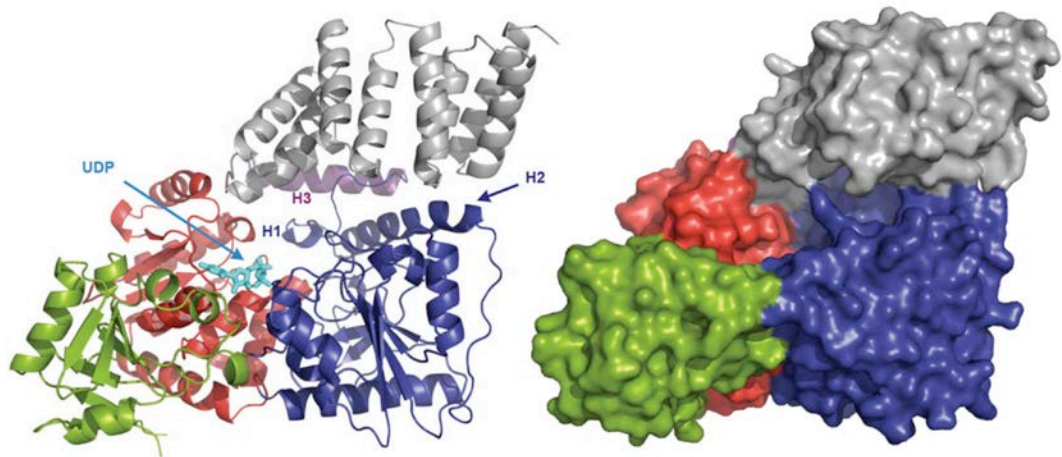


Figure 1-6 Schematic of OGT domain architecture with UDP bound. The catalytic lobes are shown in blue (N-terminal) and orange C-terminal), and the TPR domains that cap the active site are in grey, and the transitional helix linking them to the catalytic domain is coloured purple and green (Lazarus et al., 2011).

1.9.3.2 Structural Analysis of OGA

Human OGA (hOGA) is a 92-kDa enzyme that consists of three domains linked by a disordered region. One N-terminal hydrolase catalytic domain (GH84 family) (orange), a C-terminal domain that has been reported to be a histone acetyltransferase (blue) and a stalk region (green) that is interrupted by a disordered region (grey)(figure 1-7).

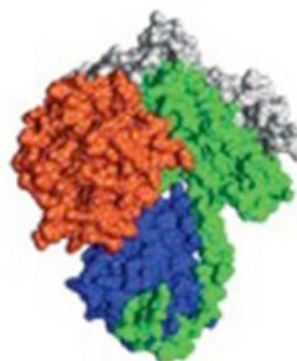


Figure 1-7 Schematic of OGA architecture. The hydrolase-catalytic domain is shown in orange, histone-acetyltransferase in blue, stalk region in green and disordered region in grey(Vocadlo, 2012).

1.9.4 Biosynthetic pathway

UDP-GlcNAc is the sugar-nucleotide donor for addition of O-GlcNAc to proteins. The synthesis of UDP-GlcNAc is a tightly regulated process. Between 2-5% of total glucose utilization is directed toward generating this sugar nucleotide (Hart et al., 1995).

Between 2-5% of the fructose-6-phosphate formed is directed to the hexosamine biosynthetic pathway (HBP), in which UDP-GlcNAc is produced. The first step in HBP is catalysed by glutamine/fructose aminotransferase (GFAT). This step is important because it links the glycolytic metabolism to amino acid metabolism via the requirement of glutamine as the amide source. Also GFAT is a rate-limiting enzyme in this pathway. This enzyme is feedback inhibited by UDP-GlcNAc. Over-expression of GFAT leads in an increase in cellular UDP-GlcNAc, hyperinsulinemia and insulin resistance. The hexosamine core then is further modified through the addition of an acetyl group, linking this pathway to fatty acid oxidation (Love and Hanover, 2005).

1.9.5 Regulation of glucose homeostasis

From the first studies became rapidly clear that the addition of O-GlcNAc is developmentally important (Love et al., 2010). Perhaps the most persuasive evidence that the addition of O-GlcNAc is a dynamic post-translational modification comes from studies where cycling of O-GlcNAc has been blocked. These studies used soluble galactosyl transferase, an enzyme that caps O-GlcNAc with a galactose residue blocking recognition by hexosaminidases (OGA). Either overexpression of this enzyme, or microinjection into the cytosol, resulted in cell cycle arrest and apoptosis. Especially the group of Tak Mak blocked the murine glucosamine-6-phosphate (EMeg32), which plays an emerging role in UDP-GlcNAc biosynthesis, and they noticed embryonic lethality at day7.5 and proliferative defect in mouse ES cells. Most of these defects can be ascribed to an impairment in the synthesis of the donor nucleotide-sugar UDP-

GlcNAc, which leads in the lack of cytosolic and Golgi-driven glycosylation(Boehmelt et al., 2000b, Boehmelt et al., 2000a).

1.9.6 Regulating protein phosphorylation and thus protein function

O-GlcNAc addition occurs at serine and threonine residues, which can be also phosphorylated. Such evidence led to the hypothesis that we have a reciprocal relationship between O-GlcNAcylation and phosphorylation.

In order to investigate this hypothesis, it is necessary to measure the level of O-GlcNAcylated proteins after extrinsic activation of phosphokinase and phosphatase and secondly after using PKC and PKA inhibitors. In the first attempt glycosylation of proteins was reduced and in the second was increased (Griffith and Schmitz, 1999). So the above data suggest that is a dynamic equilibrium between phosphorylation and O-GlcNAcylation regarding the modification of serine/threonine residues. Cell signals leading to the production/activation of phosphokinases, change the balance of the equilibrium, giving greater control to phosphorylation by “suppressing” O-GlcNAcylation.

An example of this reciprocal relationship is RNA polymerase II. The C-terminal domain of polymerase consists of tandem repeats that can be either phosphorylated or glycosylated. In vivo experiments showed that the addition of a single residue of O-GlcNAc blocked phosphorylation and the phosphorylation of residues inhibited glycosylation. The data suggest that this reciprocal relationship is necessary for the prevention of premature transcriptional initiation. The RNA pol II is hypo phosphorylated when it assembles and when the transcription is about to begin it gets hyper phosphorylated by the removal of O-GlcNAc (Conaway et al., 2000).

1.9.6.2 Interplay between O-GlcNAcylation and phosphorylation

One of the main mechanisms that underlies the biological actions of O-GlcNAc involves its interplay with protein phosphorylation. Recent advances have allowed the full elucidation of the relationship between the above post-translational modifications. Until now, four different types of interaction have been identified (Fig 1-8)

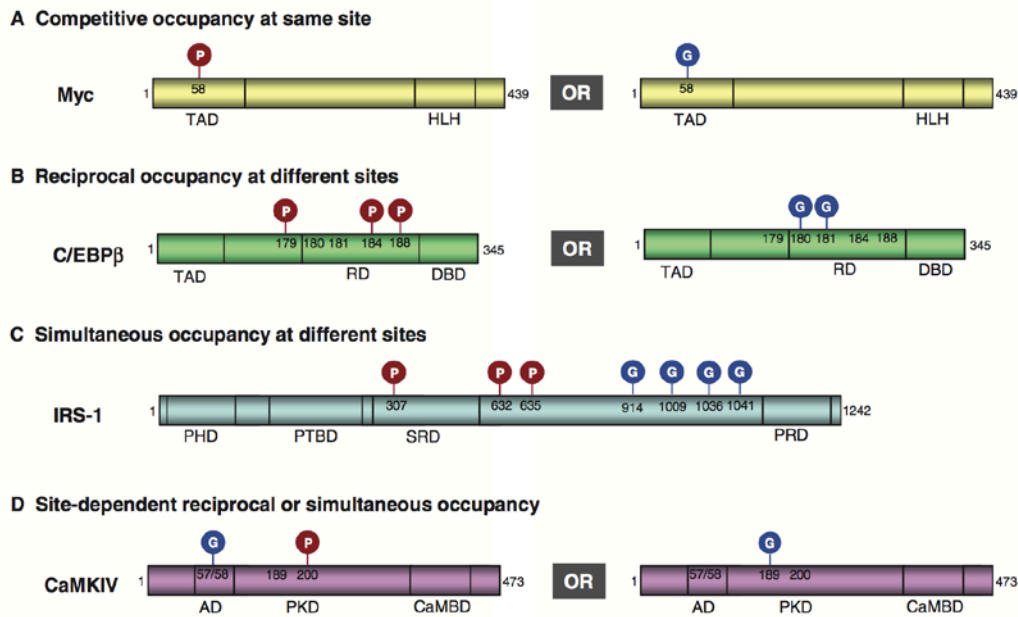


Figure 1-8 Four different types of O-GlcNAc–phosphate crosstalk on protein substrates. **A.** Alternative and competitive occupancy **B.** Alternative and reciprocal occupancy at different sites **C.** Simultaneous occupancy at different sites **D.** Site dependent reciprocal or simultaneous occupancy (Zeidan and Hart, 2010)

First, these modifications can competitively and alternatively occupy the same serine or threonine residue of a protein. Second, O-GlcNAc and phosphate can competitively and alternatively occupy different sites, which can be adjacent or distant. Third, they can simultaneously occupy different sites. In this case the balance between O-GlcNAcylation and phosphorylation determines the function of the protein. Fourth, there can be complex crosstalk involving both site-specific alternative and simultaneous occupancy of O-GlcNAc and phosphate moieties. O-GlcNAc–phosphate crosstalk participates in nearly every biological aspect of protein function: changes in subcellular localization, protein turnover, enzymatic activity, and the DNA-binding and

transactivation properties of transcription factors. These changes in the activities of specific proteins play important roles in major cellular processes (Zeidan and Hart, 2010).

1.9.7 Altering protein degradation

O-GlcNAc plays an emerging role in protein degradation in two ways. First by altering the targeting of some proteins to the proteasome and secondly by altering the activity of the proteasome itself. Some proteins contain domains that are rich in Proline, Glutamine, Serine and Threonine (PEST sequences). Phosphorylation events (activation) occurring on these residues trigger the protein degradation process. It is proposed, that the addition of O-GlcNAc, possibly blocks phosphorylation sites that regulate protein degradation. The main example is Oestrogen Receptor β (ER β). In support of this data, mutation of the main sites of glycosylation results in protein with longer half-life than the wild-type (Cheng et al., 2000, Cheng and Hart, 2001).

Oestrogen Receptor β plays a very important role in the development of several systems. Mice lacking ER β have been generated with severe effects in bone and brain development.

1.9.8 Adjusting the localization of proteins

It was initially proposed that O-GlcNAc is an alternative NLS (Nuclear Localization Signal) because for some phospho-proteins such as c-Myc, Tau, Stat5a, Pax-6, ELF-1, Sp1, mTor, the nuclear forms were more O-GlcNAc modified than the cytoplasmic forms. The concept now is changed because after the examination of this model in *C.elegans* scientists found that nuclear transport of several transcription factors was normal despite the complete absence of O-GlcNAcylation due to OGT knockdown. So, if O-GlcNAc plays a role in nuclear localization, it may not be as a NLS signal but rather as a nuclear retention signal (Hanover et al., 2005).

1.9.9 Modulating protein-protein interactions

The O-GlcNAcylation also modulates protein-protein interactions. The addition or removal of O-GlcNAc at specific residues affects the binding ability of the protein to other proteins in a positive or negative way. Three transcription factors, which are known to be affected, are Sp1, YY1, and ELF-2. Addition of O-GlcNAc may cause less effective binding of the protein to its targets. Alternatively addition of O-GlcNAc to Stat5a at Thr92 residue is necessary in order to induce gene transcription in response to prolactin stimulation (Juang et al., 2002, Hiromura et al., 2003, Gewinner et al., 2004).

1.9.10 Mediating transcription

O-GlcNAc modifies numerous transcription factors in response to glucose/glucosamine concentration change causing up- or down-regulation in gene expression. Typical example is Sp1 transcription factor (analysed below).

1.10 O-GlcNAcylation in sperm cell

As discussed, O-GlcNAc modification has many interactions with phosphorylation, which plays a huge role by regulating a vast amount of signalling pathways during capacitation. Both occur in the nucleus and cytoplasm and both modify serine and threonine residues. Both modifications are dynamic processes that cycle on their protein substrates with a variable rate. The protein, the site of modification and cellular state determine the rate of phosphorylation or O-GlcNAcylation (Hart et al., 1995).

This leads to the hypothesis that OGT and phosphokinases compete for the modification of the same residues and possibly the tightly regulated process of capacitation requires the careful orchestration of both modifications.

1.11 Objectives and research strategy

- **The aim of this work is to identify and understand the role of O-GlcNAc modified proteins during sperm capacitation**
- **Provide an insight how O-GlcNAcylation effects the process of hyperactivation**

1.12 Project Hypothesis

The main hypothesis of the project was that O-GlcNAcylation is heavily implicated and has a biological role in the process of sperm capacitation and especially hyperactivation. A series of experiments were carried out in order to elucidate the role of O-GlcNAcylation

- Western blot analysis, in an attempt to better understand how rapidly O-GlcNAcylation changes during capacitation.
- Modulation of stoichiometry by using small molecule inhibitors of OGA in order to study its function/effects in sperm capacitation
- Complete motility analysis

Chapter 2: Materials and methods

2.1 Introduction

This chapter will give an overview of all the material used in the present study, as well as detailed protocols of all the methods employed. A brief outline of the experimental procedure followed in the figure below (figure 2-1).

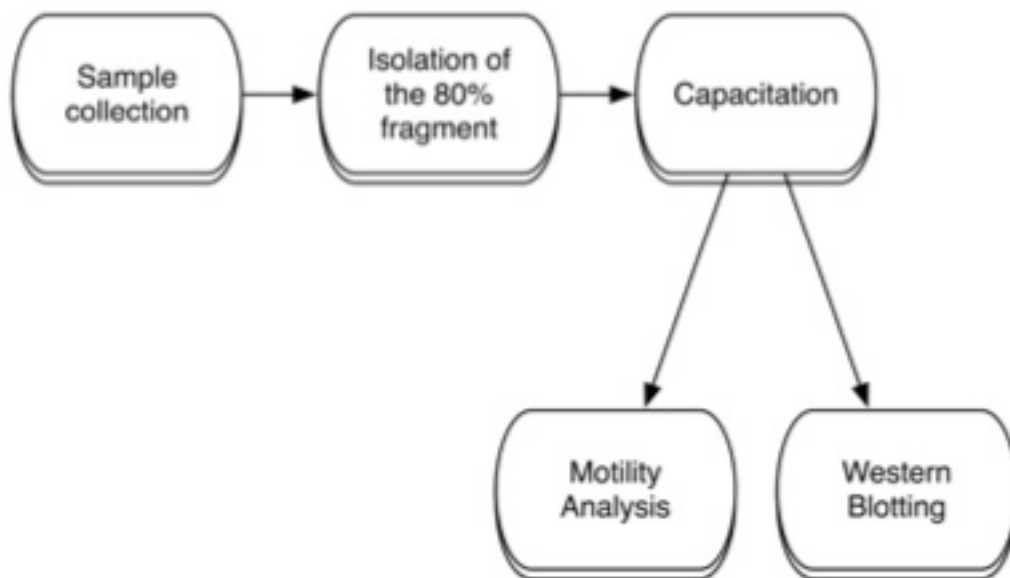


Figure 2-1: Flow chart showing the simplified experimental protocol used in the present study

2.2 Sample Collection

Semen was collected from healthy donors according to WHO (2010) criteria, taking part in the sperm donor program at Dundee University at Ninewells hospital. All donors gave informed consent that their spermatozoa would be used for research purposes only. Samples were produced by masturbation following by at least 2 days of sexual abstinence. The semen was allowed to undergo liquefaction at 37°C for 30min before further analysis.

2.3 Discontinuous Gradient Centrifugation

A 40%, 80% solution of Percoll in NCB was prepared. Firstly, 2ml of 80% was placed into a conical tube. Thereafter 2ml of 40% were carefully layered on top. 1ml of semen

was layered on top of the gradients, taking care in order not to mix the layers. The tube was centrifuged for 20min at 300x g. The centrifugation resulted into two distinct cell populations (fragments). The 40% fragment (Immature sperm) and the 80% fragment (mature sperm). The mature cells migrated to the bottom of the tube and formed a pellet, whereas the immature cells migrated between the 40% and 80% Percoll layers. The seminal plasma and debris remained in the top layer. After centrifugation the different layers were carefully removed to prevent any disturbances and mixing. The collected fractions were then washed with Non-Capacitating media to remove the Percoll and then they were re-suspended in the appropriate buffer.

2.4 Capacitation

Synthetic Tubal Fluid (STF) was used as media that supports capacitation. The media was composed of 4.7 mM KCl, 3 mM CaCl₂, 1 mM MgSO₄·7H₂O, 106 mM NaCl, 5.6 mM D-Glucose, 1.5 mM NaH₂PO₄, 1 mM Na pyruvate, 41.8 mM Na lactate, 25 mM NaHCO₃, 1.33 mM Glycine, 0.68 mM Glutamine, 0.07 mM Taurine, Non-essential amino acids (1: 100 dilution in STF) and 30mg/ml BSA. Non-Capacitating Buffer (NCB) was adapted from STF excluding amino acids and NaHCO₃. The components of NCB was 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄·7H₂O, 116.4 mM NaCl, 5.6 mM D-Glucose, 1.0 mM NaH₂PO₄, 2.7 mM Na pyruvate, 41.8 mM Na lactate and 25 mM HEPES. The pH of NCB was adjusted to 7.4 with NaOH, and pH of STF was allowed to reach about 7.4 at 37°C and 5% CO₂ before usage. The osmolality of the buffers were checked to be between 290-320 mOsm/kg.

2.5 Motility Analysis

The most common tool to study the sperm movement is high-speed video-micrography. Computer-aided semen analyzers (CASA) are based on image-analysis and by following the movement of the sperm head, can calculate parameters such as percentage of static/moving cells, flagellar beat frequency, surface of the flagellar beat envelope

and the maximal wave amplitude.

A Hamilton Thorne 'Ceros' Computer Assisted Sperm analyzer(v12) was used for the assessment of sperm kinematics in all experiments. The analyzer uses negative phase contrast trinocular optics and a high-resolution CCD camera to determine the motility parameters. . The settings used for the instrument to detect human spermatozoa were: 60 Hz; low and high size gates, 0.35 and 2.80, respectively; low and high intensity gate, 0.5 and 2 respectively; minimum number of data points, 13; non-motile head size, 6 pixels; non-motile head intensity, 160. The percentage of hyperactivated cells was assessed using standard criteria to identify hyperactivation; $VCL \geq 150 \mu\text{m/s}$, linearity $\leq 50\%$, and $ALH \geq 7$ (Mortimer et al., 1998). These are figure 2-2:

- Total motility (percentage of motile spermatozoa) will be used to report motility
- Progressive motility (percentage of progressive motile cells)
- Curvilinear velocity (VCL) ($\mu\text{m/s}$) (the time average velocity of sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope)
- Straight line velocity (VSL) ($\mu\text{m/s}$) (the time average velocity of sperm head along the straight line between its first detected position and its last)
- Average path velocity (VAP) ($\mu\text{m/s}$) (the time average velocity of sperm head along its average path)
- Amplitude of lateral head displacement (ALH) ($\mu\text{m/s}$) (the magnitude of lateral displacement of sperm head about its average path)
- Linearity (LIN) (%) (The linearity of a curvilinear path)
- Straightness (STR) (%) (Linearity of the average path)
- Beat-cross frequency (BCF); (beats/second) average rate at which the sperm's curvilinear path crosses its average path

- Rapid cells; the percentage of rapid moving cells,
- Static cells; percentage of static/motion-less cells.

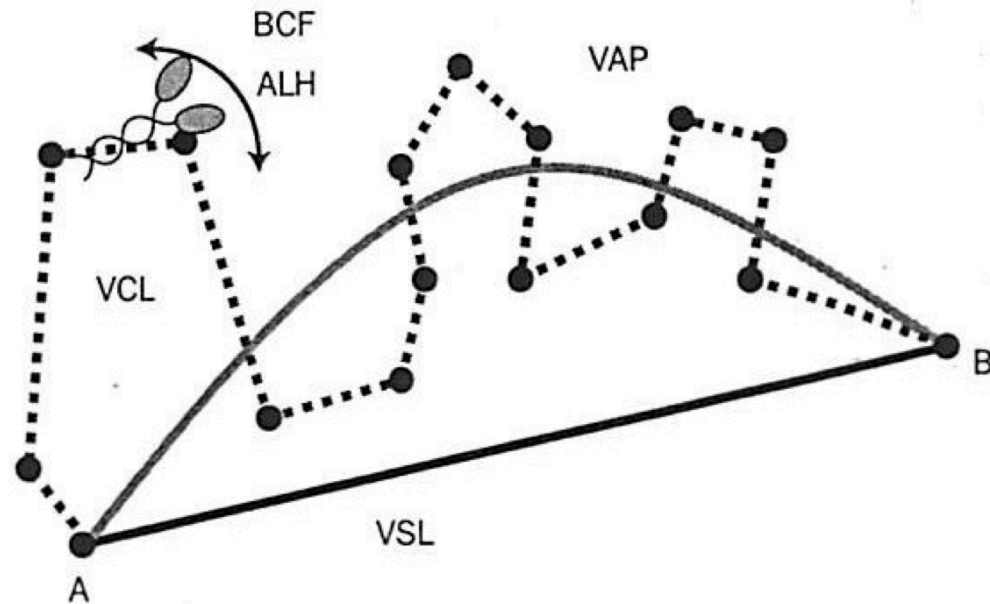


Figure 2-2 Visual presentation of the parameters analyzed by CASA (Hinting et al., 1988).

2.6 Procedure

A Hamilton Thorne 2x-cell (20 μ m depth) microscope slide and a cover slip were pre-warmed for two minutes on a Minitherm heated stage. 4 μ L of the sample was pipetted into each chamber and carefully covered with the pre-warmed cover slip in such a way to avoid drifting of the sample. To obtain representative measurement an approximate of 800 cells were assessed. The playback feature was used routinely to ensure that the majority of sperm cell were picked up, and that drift and the presence of round cell or debris were not biasing scan results. Once four lines of data, each comprising at least 200 motile cells, were obtained then the slide was removed and washed. Data was transferred from CASA to a specifically designed Excel sheet, and manipulated from there.

2.7 Protein extraction/storing

The sperm sample was centrifuged for 5 min in 17,000x g. After centrifugation, the sperm pellet was re-suspended in Laemmlesample buffer (no reducing agent) at the concentration of 0,25 million cells per μl (5 million cells into 20 μl) and the sample was heated at 95⁰ C for 5 minutes. The insoluble material was removed from the sample by centrifugation (17,000x for 5 min). The sperm extract was stored at -20 or -80°C until ready for electrophoresis.

Laemmle sample buffer recipe:

- 0,5M Tris-HCl (pH=6.8)
- 8% SDS (Sigma L4509)
- 40% Glycerol (Sigma G5S16)
- 0.008% bromophenol blue (Sigma B0126)

2.8 Western blot analysis

This procedure describes the technique by which sperm proteins can be identified on a solid support (nitrocellulose or PVDF membrane). Proteins are electrophoretically separated according to their molecular weight and transferred on a membrane where proteins will be detected using specific antibodies(Towbin et al., 1979).

2.8.1 Gel preparation

Firstthe glass plates have to be cleaned very thoroughly with regular soap and rinsedcarefully with water. Then the glasses have to be cleaned with 100% methanol or 100% acetone, to remove any remaining soap. The system is assembled carefully before pouring the acrylamide solution (Mini-Protean Tetra system Bio-Rad). It is important to wear gloves to minimize fat from the hands and also acrylamide is a powerful neurotoxin.

Acrylamide solution was prepared and the TEMED and APS (catalysts) were added just before pouring the gel solution between the glass plates. This solution is called separating gel and different concentrations could be made according the protein size of interest (7.5- 15%). The volume necessary made for 1-2 slab gels is 5 ml.

Separating Gel Recipe (10% acrylamide solution):

- Water: 2,880 μL
- Acrylamide: 1,500 μL
- Tris-HCL (ph=8.8): 1,500 μL
- SDS 20%: 30 μL
- APS: 60 μL
- TEMED: 6 μL

Next we pour the gel solution using a Pasteur pipette and n-Butanol (water-saturated) is overlaid on the top of the gel ($\sim 100 \mu\text{l}$). The limit of the top end (separating gel) is determined at 1 cm under the wells. The gel should polymerize in 10-20 min. We can determine when the polymerization is completed, by looking the extra gel left in the pipette or to see if the upper corners are rounded. Once the separating is polymerized, the stacking gel is overlaid on the top of the first gel and the comb was inserted after removing the Butanol layer. The thickness of the gel could change the volume needed, in this situation 0.75 mm is used.

Stacking Gel Recipe (4% acrylamide solution):

- Water: 3,125 μL
- Acrylamide: 500 μL
- Tris-HCL (pH=8.8): 1,250 μL
- SDS 20%: 25 μL
- APS: 50 μL
- TEMED: 5 μL

2.8.2 Sample preparation

The sample is prepared as following. Usually, the samples are frozen into 2X sample buffer. We warm the stock solution to be sure the SDS is not precipitated. The volume used per lane is usual 10-25 μ l and Dithiothreitol (DTT) was used to reduce proteins. Before loading the samples, the wells can be washed with the running buffer (Tris-Glycine-SDS) and the protein samples were heated at 95°C (3-5 min). The tank was filled with the 1X Tris-Glycine-SDS and the gel is running under constant current (20 mA/gel, 200 V limit).

Running buffer recipe (1L, 1x)

- Trizma base (Sigma T1503): 3.03gr
- Glycine (Sigma G8898): 14.4gr
- SDS (Sigma L3771): 1gr

2.8.3 Electrophoresis

At the beginning the voltage is ~115 V, but after ~20 min the condition switch to voltage constant because the voltage reaches 200V and the migration is stopped when the dye front is close to get outside of the gel (approximately 1.5 h).

2.8.4 Protein Transfer

Next to the protein separation, the gel is carefully removed from the glass plates and soaks briefly in the transfer buffer. Therefore, gel is assembled in sandwich between Whatman 3M paper (2 pieces each side) including nitrocellulose (or PVDF) membrane. The membrane and pieces of paper was just the size of the gel (no extra) and the stacking gel is not usually kept for transfer. Importantly, the membrane must be at the positive side of the sandwich. When the transfer cassettes are in place in the tank filled

of transfer buffer the proteins are transferred for 2h at constant voltage (50V) for 2 hours at room temperature with constant agitation (magnetic bar).

Transfer buffer recipe (1L, 1x):

- Trizma base (Sigma T1503): 3.03gr
- Glycine (Sigma G8898): 14.4gr
- Methanol (Sigma 179337): 20%

2.8.5 Blocking and Incubation with the Primary antibody

Once the transfer is completed the membrane is trimmed as the gel size if necessary and standard molecular weights are identified with a pen in case if during the incubation the staining is lost. At this point the membrane could be incubated in a blocking solution (5% milk powder in TBS-T) in a 50 ml falcon tube for an hour with constant agitation (rolling). Then, the membrane is rinsed with TBS-T and incubates at 4°C overnight with the primary antibody diluted in the blocking solution with constant agitation (rolling).

2.8.6 Incubation with the secondary Antibody

The next day, the membrane is washed 3 times with 5 ml of TBS-T for 10 minutes every time. The primary antibody bound to proteins is detectable by incubating the membrane with a secondary antibody conjugated to peroxidase (HRP-goat anti mouse or goat anti-rabbit). The secondary is incubated with the membrane for 60 min at room temperature. The same washes as for the primary antibody are performed in TBS-T.

Antibodies list:

Ab raised against	Species	Isotype	Company	Concentrations
1) O-GlcNAc	Mouse mAb	IgG1	Abcam –ab2739	1/250
2) Anti-phosphotyrosine	Mouse mAb	IgG2	Millipore-16-452	1/1000
3) β -actin	Rabbit poly-clonal	IgG	Abcam –ab8227	1/1000
4) Anti-mouse IgG HRP linked	Horse	IgG	Cell Signaling# 7076	1/500
5) Anti-Rabbit IgG HRP linked	Goat	IgG	Cell Signaling# 7074	1/500

Table 2-3: Description of the type and concentrations of the antibodies used in westerns**2.8.7 Visualization**

In the last step prepare a mixture of peroxidase and luminol (50-50) as a developer solution (1 ml) and pipet the solution directly on the membrane and leave for 5 minutes. After the 5 minutes we transfer the membrane in the blot holder and wipe any liquid left with a paper towel. Liquid must not come into contact with the film. In the processor room place the film over the blot starting with 1 minute and 5 minutes of exposure.

2.9 Densitometry

Image J was used to compare the density (intensity) of the bands of the western blot. A flatbed scanner was first used to scan the x-ray film. The method used is described in the ImageJ documentation.

2.10 Statistical analysis

Sperm motility data were analyzed using the independent Student t- test. Motility data are presented as mean \pm standard error of the mean (SEM). The significance level was set as $p < 0.05$.

Chapter 3: Study of O-GlcNAcylation during capacitation

3.1 Introduction

Mammalian sperm are unable to fertilize the oocyte immediately after ejaculation but they must undergo a cascade of biochemical and physiological changes that facilitates the development of the fertilization competent state. Spermatozoa during capacitation modify their motility pattern from a progressive state to a hyper-activated state. Progressive state is characterized by high flagellar beat frequency with low bend angle of the tail. On the other hand, hyper-activated state involves deep flagellar bends, due to increased flexure of the mid-piece and a decreased beat frequency resulting in greater lateral displacement of the head. Acquisition of the hyper-activated state by the spermatozoa has been reported for all eutherian spermatozoa (Suarez and Osman, 1987) and is essential for the effective penetration of the cervical mucus and penetration of the outer layers of the oocytes, leading to a successful fertilization. Not all the molecular mechanisms and signal transduction pathways involved are clearly known and understood, but various studies have shown that pH (Parrish et al., 1989) and cAMP are important regulatory components (Marquez and Suarez, 2007). Numerous studies also report the role of other PTMs like tyrosine/serine/threonine phosphorylation in human sperm capacitation but nothing has been ever reported about the involvement of O-GlcNAcylation as a possible regulator of capacitation. In this part of the project, we have used anti-O-GlcNAc antibody (RL2) in human sperm, to detect proteins that serve as substrates for OGT. We report multiple sets of O-GlcNAcylated proteins in the molecular weight range of 25kDa to 250kDa. The O-GlcNAcylation levels appear to be decreasing during the capacitation process. This fact suggests a vital role of O-GlcNAc modification for the acquisition of the hyper-activated state, which may involve the dynamic interaction between O-GlcNAcylation and ser/thr phosphorylation.

3.2Results

In our attempt to identify and understand the role of O-GlcNAc modification and how rapidly changes during capacitation, a time-course experiment was performed. Due to high amount of sperm protein required in each lane in order to detect the modification, 3 individual donor samples were pooled into one sample. The 80% fragment was isolated and incubated under capacitating conditions for 4h. At the time-points of 0h, 0.5h, 1h, 2h, 4h protein was extracted and subjected to Western blotting. Protein analysis for 3 different antibodies was performed. The first antibody is a RL2 (anti-O-GlcNAc antibody). The second antibody is an anti-phosphotyrosine antibody. Tyrosine phosphorylation is the most commonly used marker, in order to evaluate the progress of capacitation. Finally β -actin antibody was used as a loading control (figure 3-1).

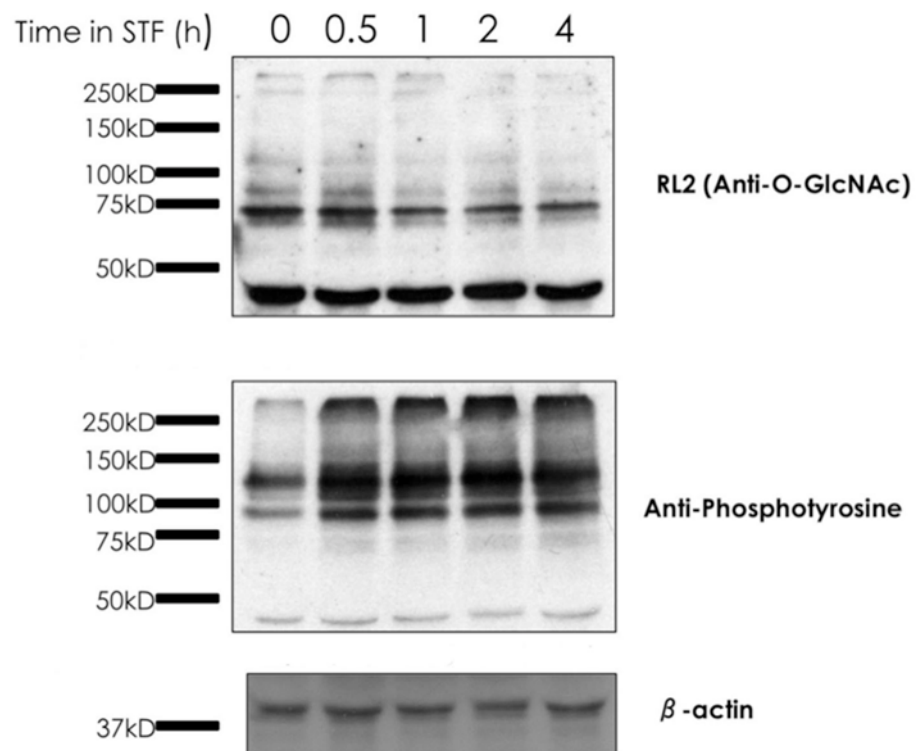


Figure 3-1: Time-course of protein O-GlcNAc levels in ejaculated human sperm under conditions that support capacitation. At the times listed, sperm proteins were extracted and subjected to SDS-PAGE immuno-blotting with an anti-O-GlcNAc monoclonal antibody. Three individual donor samples have been pooled in order to perform the above experiment. The results show that O-GlcNAcylation levels decrease during capacitation. Tyrosine phosphorylation increases during the same time period, revealing that the process of capacitation is progressing normally. Finally the level of β -actin remains stable (loading control)

The results reveal that O-GlcNAcylation levels decrease markedly following capacitation. There was a time-dependent decrease in O-GlcNAcylation of a subset of proteins of Mr =40-250 kDa.

Discussion

In the study presented, several O-GlcNAc proteins were identified, with the most prominent migrating at Mr=40-250kDa. The level of O-GlcNAcylation appears to decrease markedly following capacitation (Figure 3-1). Un-capacitated sperm demonstrated a high level of O-GlcNAcylation on Serine/Threonine residues compared with sperm that were incubated for 4h in capacitating media containing bicarbonate. As shown in Figure 3-1, when sperm were incubated for various periods of time between 0 and 4h in media that supports capacitation, there was a time-dependent decrease in O-GlcNAcylation of a subset of proteins of Mr =40-250kDa. While two immune-reactive bands appeared under all conditions (40 and 70kDa), the remaining bands detected with the anti-O-GlcNAc antibody appeared to be modified in a time-dependent manner.

Chapter 4: Inhibition of O-GlcNAcylation under capacitating conditions

4.1 Introduction

Sperm dysfunction is the single most common cause of infertility and accordingly to the most recent studies affects 1 in 15 men (HFEA 2005, www.hfea.gov.uk). Very little is known about the factors that cause sperm dysfunction due to lack of understanding of sperm physiology. Therefore, in order to develop drugs to cure male infertility, there is an urgent need to develop drugs capable of improving the fertilization capacity of the man in order to minimize the usage of ART, especially ICSI. ICSI treatment has been associated with birth defects and the use of it, has been increased dramatically, while the proportion of patients diagnosed with male factor, is stable (Davies et al., 2012, Oehninger, 2011, Jain and Gupta, 2007). Elucidating the sperm physiology will allow us to overcome the obstacle of ART and develop less invasive treatments. Sperm cells have no endoplasmic reticulum and minimal amount of cytoplasm and highly compact genetic material and they are transcriptionally and translationally inactive. Therefore control of cellular processes has to rely on modifying and changing activity of already existing proteins or enzymes. The O-GlcNAc modification is found on many sperm proteins, and addition and removal, can occur multiple times during the lifetime of a protein, imparting possible dynamic signaling properties to O-GlcNAc. Moreover, the interplay of O-GlcNAcylation and phosphorylation gives O-GlcNAc the potential to be involved in many cellular processes. In this chapter I modulate the stoichiometry of O-GlcNAcylation on proteins, in order to study its function/effects in sperm motility. One of the most direct approaches of increasing global O-GlcNAc levels is by using small-molecule inhibitors of O-GlcNAcase (OGA). The use of inhibitors of OGA in a cellular context results in a time-dependent increase in O-GlcNAc-modified proteins due to the continued activity of OGT and the impeded action of OGA. Over 12 OGA inhibitors have been described to date (Macauley and Vocadlo, 2010). In this part of the project

we examine the effects of 3 different OGA inhibitors (PUGNAc, Thiamet-G, GlcNAcstatin) and one OGT inhibitor(4Ac5S-GlcNAc)in sperm motility. Inducing O-GlcNAcylation with GlcNAcstatin (GNS) leads to lower hyperactivation levels (Sort3), Average Path Velocity (VAP), Linearity (LIN), Curvilinear velocity (VCL) and Amplitude of lateral head Displacement (ALH) in GNS. Treatment with Thiamet G reduces the percentage of progressive motile cells, hyperactivated cells (Sort3) and also reduces Linearity. Treatment with PUGNAc leads to lower hyperactivation levels (Sort 3) and linearity.Finally treatment with 4Ac5s-GlcNAc, which is an OGT inhibitor significantly changes the percentage of progressive motile cells and Linearity. Western blots of the above specimens confirm the PTM induction/reduction in a protein level.

4.2 Methods

The 80% fragment was isolated as described in Chapter 2. The sample was separated and incubated in capacitating media (STF) with DMSO (vehicle control) or the appropriate concentration of the inhibitor for 4h. At the end of the capacitating period motility measurements were taken, followed by protein extraction for western blot analysis.

Drug Concentrations	
GlcNAcstatin	1 μ M
Thiamet G	1 μ M
PUGNAc	100 μ M
4Ac5S-GlcNAc	10 μ M

4.3 Motility Results

In Chapter 3 the results revealed that O-GlcNAc is present in many sperm proteins (protein bands between 40-250 kDa) and is constantly decreasing when cells are incubated under capacitating conditions in presence of bicarbonate. In this part of the project I modulate the stoichiometry of O-GlcNAcylation, by using highly potent inhibitors, in order to study it's effect on sperm motility, because hyper-activated state is regulated by a series of phosphorylation events and O-GlcNAc is proven to interact with phosphorylation events in other cell types.

4.3.1 CASA motility analysis overview

The settings and conditions used to evaluate sperm motility parameters are all described in chapter 2.5. The total population of sperm cells can be divided into two sub-populations, motile and static. The static-cell population consists of non-motile cells with minimum and maximum size values for movement as defined by CASA settings [for humans: 0.35(min) - 2.8(max)]. The comparison of the static cell population between treated and untreated cells provides the necessary information about the toxicity of the compounds (drugs). Since there is no significant difference in all treatments (figure 4-1,7,13,19), no toxic effects can be attributed to the compounds used to treat the cells. The motile cell population can be further divided into 3 categories (rapid, medium, slow). Slow cells are defined as cells with either of the following attributes: $VAP < VAP \text{ CUT-OFF} = 5 \mu/s$ or $VSL < VSL \text{ CUT-OFF} = 11 \mu/s$. Medium cells are moving with $VAP: \text{CUT-OFF}(5 \mu/s) < VAP < \text{PATH-VELOCITY}(25 \mu/s)$. Finally rapid cells are moving with $VAP > \text{PATH-VELOCITY}(25 \mu/s)$. No significant differences in all three cases were observed (figure 4-2,8,14,20). The next charts compare the percentage of progressive cells between treated and non-treated populations (figure 4-3,9,15,21). Progressive cell population is defined as cells moving with both $VAP > \text{PATH VELOCITY}(25 \mu/s)$ and $STR > 80\%$ as determined by values defined for humans in the ANALYSIS SETUP. No significant differences between the percentages of progressive cells in all three treatments. On the next two charts I compare the VAP and VSL values along with their frequency distributions. Only GNS reduced significantly VAP. No significant differences were observed in VSL (GNS treatment) and VAP, VSL (rest of the treatments) (figure 4-4,10,16,22). Next I compare the level of hyper-activated cells between treated and non-treated populations (figure 4-5,11,17,23). Hyper-activated cells are cells defined as cells moving with both three parameters: $VCL \geq 150 \mu/s$, $ALH \geq 7 \mu$, $LIN \leq 50$. Only treatment with GNS causes

significant decrease in hyperactivation levels. Treatment with Thiamet G and PUGNAc causes a decrease in hyper-activation levels that appears not to be significant, probably due to small sample population. In my attempt to investigate which of the parameters of the hyper-activated cells is affected the most, I compared the average values of VCL, LIN and ALH along with their frequency distributions (figure 4-6,12,18,24). In every case, the amplitude of lateral head displacement was significantly decreased and the rest of the hyper-activation parameters remained unaffected for Thiamet G and PUGNAc. The average value of VCL was significantly decreased in GNS treatment.

4.4 Statistics

4.4.1 Inhibitor: GNS

10 individual donor ejaculates (N=10) were examined. The data were first tested for normality with Shapiro-Wilk test. The Shapiro-Wilk test, proposed in 1965, calculates a W-Statistic that tests whether a random sample comes from a normal distribution. If the W statistic is significant, then the hypothesis that the respective distribution is normal should be rejected. The null hypothesis for this test is that the data are normally distributed. The Prob.<W value listed in the output (Table i) is the p-value. If the chosen alpha level is 0.05 and the p-value is less than 0.05, then the null hypothesis that the data are normally distributed is rejected. If the p-value is greater than 0.05, then the null hypothesis is not rejected. The software used, was Graphpad-Prism v6.0. Furthermore, since the data are continuous and we want to compare the differences between two treatments on the same sample, a paired T-test analysis was performed. Testing for normality is a prerequisite for the paired t-Test, as all the parametric tests require that the data are normally distributed. Complete motility analysis revealed, that induction of O-GlcNAcylation with GNS causes significant decrease in Average Path Velocity (VAP) $p=0.0462$, Curvilinear velocity (VCL) $p=0.0030$, hyperactivation levels (Sort3) $p=0.0013$, Linearity (LIN) $p=0.0225$ and Amplitude of lateral head displacement (ALH) $p=0.0043$.

Donor	VAP		VCL		Sort3		LIN		ALH	
	DMSO	GNS	DMSO	GNS	DMSO	GNS	DMSO	GNS	DMSO	GNS
151	56.53	52.60	93.95	87.08	6.09	3.01	48.00	46.00	4.53	4.63
218	71.05	73.20	113.85	116.73	10.68	9.43	53.25	54.50	4.38	4.55
222	60.60	53.28	101.63	88.50	7.96	4.17	47.25	46.00	5.00	4.48
227	71.43	67.05	116.38	108.50	13.06	9.19	54.00	52.75	4.68	4.43
225	68.05	65.45	119.00	105.10	15.24	7.40	46.50	52.50	5.43	4.53
214	88.53	79.88	130.60	114.38	11.10	8.46	59.00	62.75	4.80	4.08
333	88.38	82.48	131.55	116.08	12.68	6.74	58.75	64.00	5.00	4.08
180	99.35	92.98	151.25	127.38	21.39	7.44	60.00	68.25	5.10	4.18
189	107.38	95.85	162.88	132.73	22.57	9.56	60.25	68.75	5.48	4.43
239	88.00	95.85	138.78	137.43	19.39	10.41	57.25	66.50	5.03	4.73
Shapiro-Wilk Normality test										
W	0.9438	0.9172	0.9792	0.9449	0.9455	0.9041	0.8617	0.8874	0.9575	0.909
P-Value	0.5965	0.3342	0.9607	0.6083	0.6154	0.2429	0.0799	0.1584	0.7572	0.274
Passed Normality Test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Average	79.93	75.86	125.99	113.39	14.02	7.58	54.43	58.20	4.94	4.41
S.E.M.	5.33	5.16	6.77	5.34	1.76	0.76	1.73	2.81	0.11	0.07
Paired T Test		0.0462		0.0030		0.0013		0.0225		0.0043

Table i

4.4.2 Inhibitors: Thiamet G, PUGNAc, 4Ac5S-GlcNAc (4AC)

7 individual donor ejaculates (N=7) were examined. The data were first tested for normality with Shapiro-Wilk test. The software used, was Graphpad-Prism v6.0. Then a paired T-test analysis was performed. Complete motility analysis revealed, that induction of O-GlcNAcylation with Thiamet G causes significant decrease in Progressive motility $p=0.0167$, Hyperactivation levels (Sort3) $p=0.0015$ and Linearity (LIN) $p=0.0027$. Treatment with PUGNAc significantly decreased hyperactivation levels (Sort3) $p=0.0086$ and Linearity (LIN) $p=0.0287$. Finally treatment with 4AC significantly increased Progressive motility $p=0.0093$ and Linearity (LIN) $p=0.0057$.

Donor	Progressive		Sort3		LIN	
	DMSO	Thiamet G	DMSO	Thiamet G	DMSO	Thiamet G
151	52.68	49.41	9.00	5.24	52.25	52.50
214	63.18	68.37	11.10	8.56	59.00	62.00
225	43.32	50.14	15.24	8.73	46.50	51.50
333	64.24	68.14	12.68	10.89	58.75	61.75
180	72.58	82.74	21.39	17.56	60.00	63.75
189	72.96	83.74	22.57	15.24	60.25	64.50
239	68.93	80.65	19.39	12.44	57.25	64.00
Shapiro-Wilk normality test						
W	0.8896	0.851	0.9317	0.9771	0.7992	0.7577
P value	0.2725	0.1256	0.5652	0.9444	0.1402	0.1253
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes
Average	62.56	69.03	15.91	11.24	56.29	60.00
S.E.M.	4.14	5.52	2.00	1.60	1.93	2.10
Paired T Test		0.0167		0.0015		0.0027

Table ii

Donor	Sort3		LIN	
	DMSO	PUGNAc	DMSO	PUGNAc
151	9.00	5.95	52.25	51.75
214	11.10	6.16	59.00	62.25
225	15.24	6.92	46.50	53.50
333	12.68	12.82	58.75	58.50
180	21.39	16.04	60.00	62.25
189	22.57	14.81	60.25	65.50
239	19.39	17.51	57.25	61.00
Shapiro-Wilk normality test				
W	0.9317	0.8545	0.7992	0.9202
P value	0.5652	0.135	0.1402	0.4707
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
Average	15.91	11.46	56.29	59.25
S.E.M.	2.001724169	1.887572115	1.926806953	1.889822365
Paired T Test		0.00867057		0.028723753

Table iii

Donor	Progressive		LIN	
	DMSO	4ac	DMSO	4ac
151	52.68	52.32	52.25	54.50
214	63.18	67.16	59.00	61.25
225	43.32	44.72	46.50	48.00
333	64.24	69.76	58.75	60.25
180	72.58	78.96	60.00	62.50
189	72.96	82.18	60.25	67.00
239	68.93	74.94	57.25	61.00
Shapiro-Wilk normality test				
W	0.8896	0.9145	0.7992	0.9108
P value	0.2725	0.4276	0.1402	0.4015
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
Average	62.56	67.15	56.29	59.21
S.E.M.	4.140465945	5.245469181	1.926806953	2.328925623
Paired T Test		0.00932792		0.005714357

Table iv

4.5 Inhibitor 1: GlcNAcstatin (GNS)

GlcNAcstatin is a competitive inhibitor of enzymes of the O-GlcNAcase family and binds directly to the O-GlcNAcase active site. This compound is able to raise O-GlcNAc levels in human HEK 293 and SH-SY5Y neuro-blastoma cells lines and thus provides a novel and highly potent tool for the study of the role of the O-GlcNAc in intracellular signal transduction pathways (Dorfmueller et al., 2006, Macauley and Vocadlo, 2010). The concentration used, was 1 μ M and the cells were incubated under capacitating conditions for 4 hours. At the end of the capacitating period, motility measurements were taken.

In the first chart (figure 4-1), I compare the static/motile cell population between treated and untreated cells. The static cell population consists of non-motile cells with size values defined by CASA. The CASA uses defined size values to avoid counting other cells types, that can be found in semen, such as white or red blood cells.

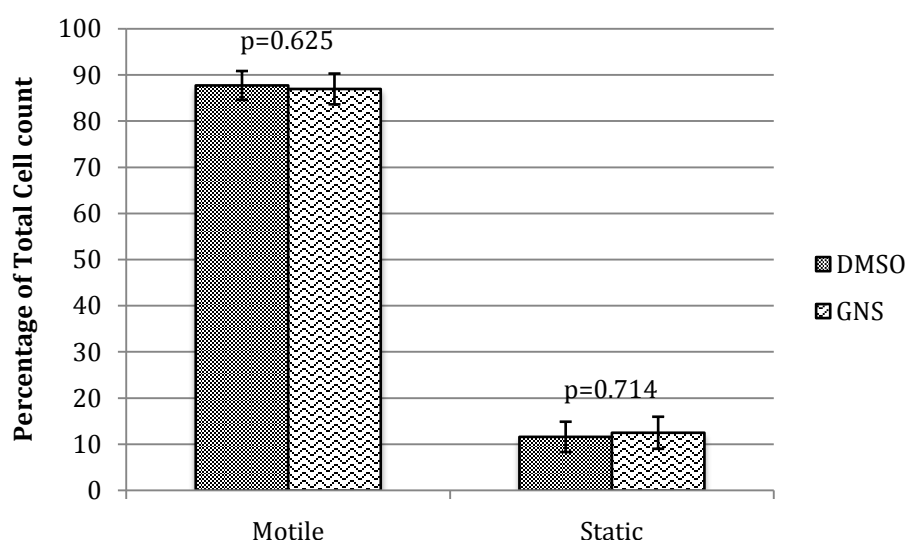


Figure 4-1 Influence of GNS treatment on human sperm motility. The histograms show that treatment with GNS doesn't affect the percentage of the motile or static cell population. Values are mean \pm SEM for 10 individual donor ejaculates.

The comparison of the static cell population between treated and untreated cells provides the necessary information about the toxicity of the compound. Since there is no significant difference, no toxic effects can be attributed to the compound used to treat the cells.

In order to be more specific, the percentage of the static cell population in untreated cells was 11.64% vs. 12.53% in the treated population. The p-value was 0.714, so no significant difference can be attributed to the result. On the other hand, the motile cell population in untreated cells was 87.75% vs. 86.98% in the treated population. The p-value was 0.625, so no significant difference can be attributed to the result.

The motile cell population can be further divided into 3 categories (rapid, medium, slow). Slow cells are defined as cells with either of the following attributes: $VAP < VAP \text{ CUT-OFF} = 5 \mu/s$ or $VSL < VSL \text{ CUT-OFF} = 11 \mu/s$. Medium cells are moving with $VAP \text{ CUT-OFF} (5 \mu/s) < VAP < \text{PATH-VELOCITY} (25 \mu/s)$. Finally rapid cells are moving with $VAP > \text{PATH-VELOCITY} (25 \mu/s)$.

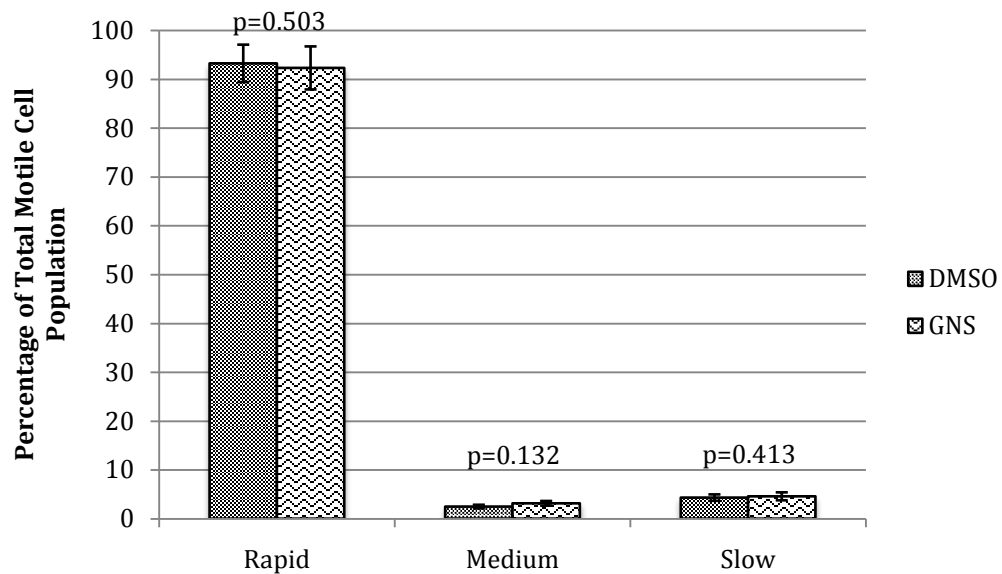


Figure 4-2 Influence of O-GlcNAcylation induction (GNS treatment) on the motile cell population. The histograms show that treatment with GNS doesn't affect the percentage of the Rapid/medium/slow cell population. Values are mean \pm SEM for 10 individual donor ejaculates

No significant differences in all three cases were observed (figure 4-2).

The percentage of the rapid cell population in untreated cells was 93.21% vs. 92.28% in the treated population. The p-value was 0.503, so there is no significant difference. Next, the percentage of the medium cell population in untreated cells was 2.47% vs. 3.14% in the treated population. The p-value was 0.132, so no significant difference can be attributed to the result. Finally, the percentage of the slow cell population in untreated cells was 4.31% vs. 4.57% in the treated population. The p-value was 0.413, so no significant difference can be attributed to the result.

The next chart compares the percentage of progressive cells between treated and untreated populations (figure 4-3). Progressive cell population is defined as cells moving with both VAP>PATH VELOCITY (25 μ /s) and STR>80% as determined by values defined for humans in the CASA ANALYSIS SETUP.

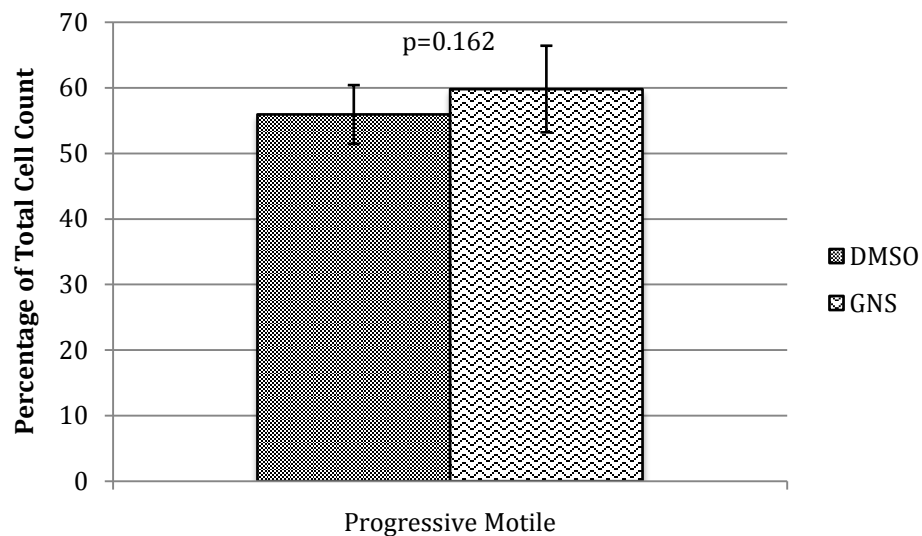


Figure 4-3: Influence of O-GlcNAcylation induction (GNS treatment) on human sperm progressive motility. The histograms show that treatment with GNS doesn't affect the percentage of the progressive cell population. Values are mean \pm SEM for 10 individual donor ejaculates.

The percentage of progressive cells in the untreated population was 55.92% vs. 59.80% in the treated population. The p-value was 0.162, so no significant difference can be attributed to the result.

On the next two charts I compare the VAP and VSL values.

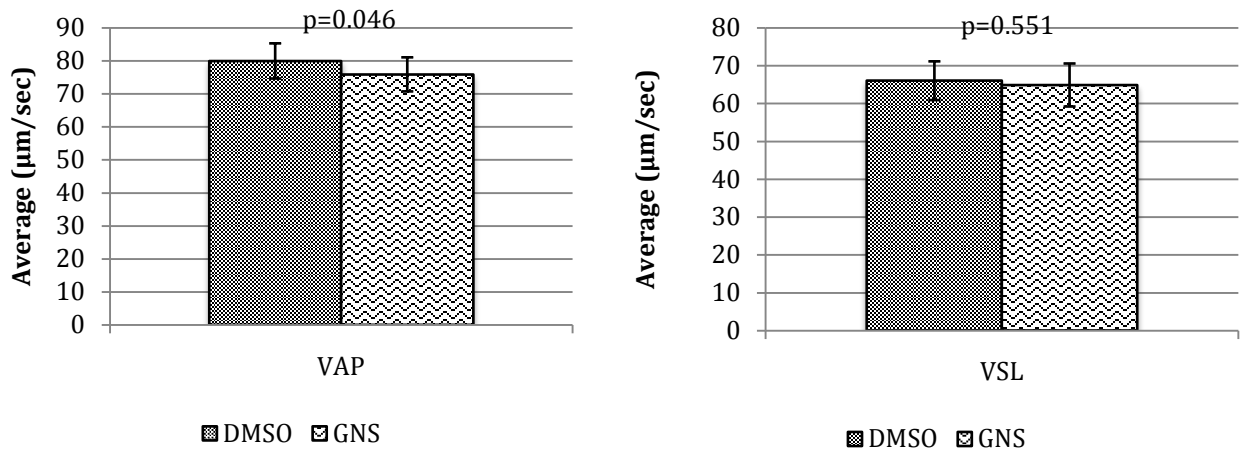


Figure 4-4: Influence of O-GlcNAcylation induction (GNS treatment) on human sperm path velocity (VAP) and progressive velocity (VSL). The histograms indicate, that treatment with GNS significantly decreases VAP but has no effects in VSL. Values are mean \pm SEM for 10 individual donor ejaculates

The average path velocity in untreated cells was 79.93 µm/sec and in treated cells was 75.86 µm/sec. The p-value was 0.046, so treatment with GNS significantly reduces the average path velocity of the cells. The average progressive velocity in untreated cells was 65.99 µm/sec vs. 64.86 µm/sec in treated cells. The p-value was 0.551, so no significant difference can be attributed to the difference in VSL.

In the next chart, I compare the level of hyper-activated cells between treated and untreated populations (figure 4-5). Hyper-activated cells are cells defined as cells moving with both three parameters: $VCL \geq 150 \mu\text{m/s}$, $ALH \geq 7 \mu\text{m}$, $LIN \leq 50$.

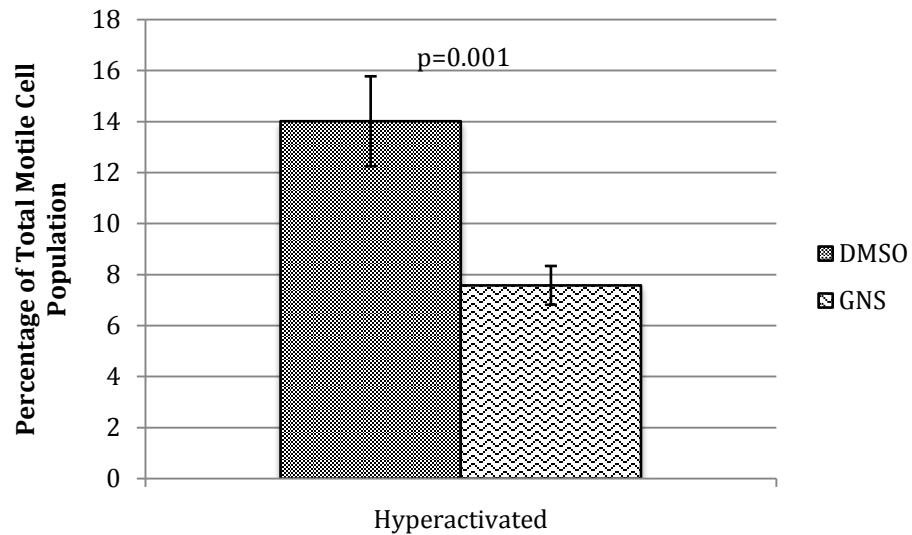


Figure 4-5: Influence of O-GlcNAcylation induction (GNS treatment) on human sperm hyperactivation levels. The histogram shows, that treatment with GNS, significantly reduces the percentage of hyper-activated cells. Values are mean \pm SEM for 10 individual donor ejaculates.

In the un-treated population the percentage of hyper-activated cells was 14.02% vs. 7.58% in the treated population. So treatment with GNS significantly reduces hyper-activation levels.

In my attempt to investigate which of the parameters of the hyper-activated cells is affected the most, I compared the average values of VCL, LIN and ALH

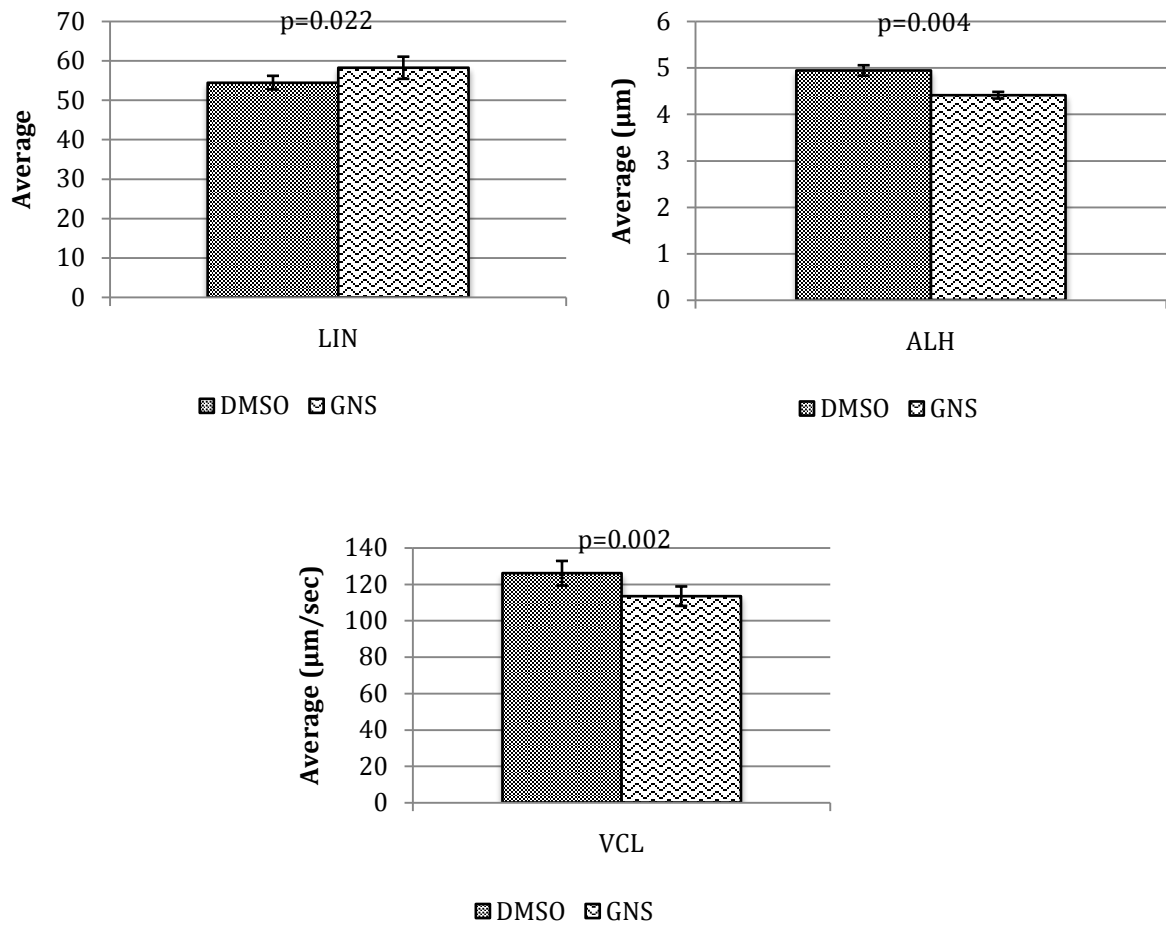


Figure 4-6: Influence of O-GlcNAcylation induction (GNS treatment) on human sperm linearity levels (LIN), amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL). The histograms show, that treatment with GNS, significantly increases LIN and decreases ALH and VCL. Values are mean \pm SEM for 10 individual donor ejaculates.

Treatment with GNS significantly affects all three parameters. The last result indicates that the lower hyper-activation levels measured in GNS treatment (figure 4-5) are the result of changes in all three parameters, which contribute to hyper-activation (figure 4-6).

4.6 Inhibitor 2: Thiamet G

Thiamet G is a potent and selective inhibitor of O-GlcNAcase and demonstrates a K_i value of 21nM. It increases cellular O-GlcNAc modified proteins. Thiamet G is the first highly potent O-GlcNAcase inhibitor known to be orally bioavailable and effectively cross the blood brain barrier.

The concentration used, was 1 μ M and the cells were incubated under capacitating conditions for 4 hours. At the end of the capacitating period, motility measurements were taken.

In the first chart (figure 4-7), I compare the static/motile cell population between treated and untreated cells. The static cells population consists of non-motile cells with size values defined by CASA. The CASA uses defined size values to avoid counting other cells types that can be found in semen, such as white or red blood cells.

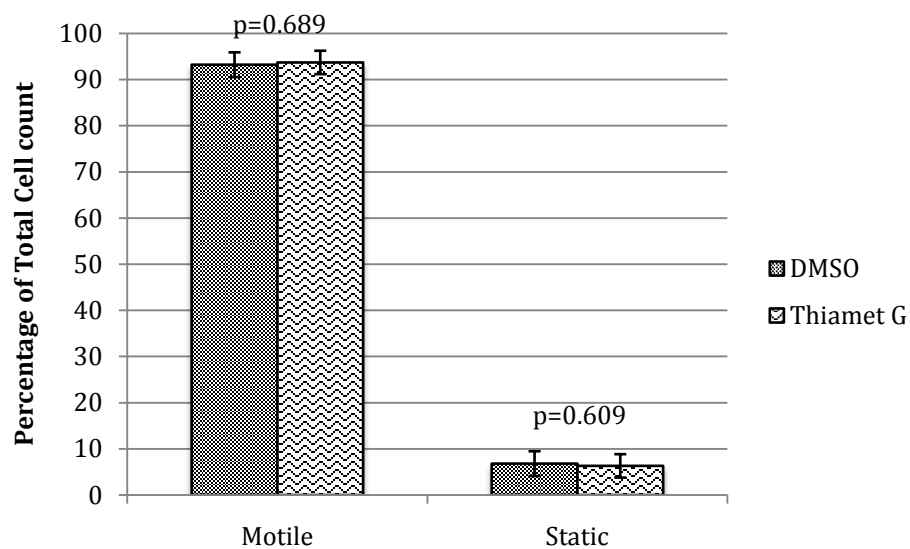


Figure 4-7: Influence of Thiamet G treatment on human sperm motility. The histogram shows that treatment with Thiamet G has no effects in the motile or static cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

The comparison of the static cell population between treated and untreated cells (figure 4-7) provides the necessary information about the toxicity of the compound. Since there is no significant difference, no toxic effects can be attributed to the compound used to treat the cells.

In order to be more specific, the percentage of the static cell population in untreated cells was 6.80% vs. 6.32% in the treated population. The p-value was 0.609, so no significant difference can be attributed to the result. On the other hand, the motile cell population in untreated cells was 93.20% vs. 93.68% in the treated population. The p-value was 0.689, so no significant difference can be attributed to the result (figure 4-7).

The motile cell population can be further divided into 3 categories (rapid, medium, slow). Slow cells are defined as cells with either of the following attributes: $VAP < VAP \text{ CUT-OFF} = 5 \mu/s$ or $VSL < VSL \text{ CUT-OFF} = 11 \mu/s$. Medium cells are moving with $VAP \text{ CUT-OFF} (5 \mu/s) < VAP < \text{PATH-VELOCITY} (25 \mu/s)$. Finally rapid cells are moving with $VAP > \text{PATH-VELOCITY} (25 \mu/s)$.

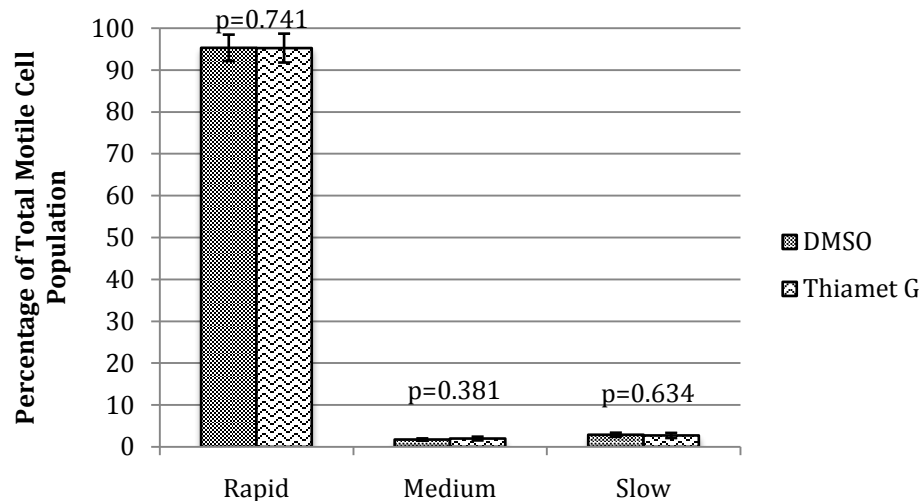


Figure 4-8: Influence of O-GlcNAcylation induction (Thiamet G) on the motile cell population. The histogram shows that treatment with Thiamet G has no effects in the Rapid/medium/slow cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

No significant differences in all three cases were observed (figure 4-8).

The percentage of the rapid cell population in untreated cells was 95.32% vs. 95.26% in the treated population. The p-value was 0.741, so there is no significant difference. Next, the percentage of the medium cell population in untreated cells was 1.76% vs. 1.99% in the treated population. The p-value was 0.381, so no significant difference can be attributed to the result. Finally, the percentage of the slow cell population in untreated cells was 2.90% vs. 2.73% in the treated population. The p-value was 0.634, so no significant difference can be attributed to the result (figure 4-8).

The next chart compares the percentage of progressive cells between treated and untreated populations (figure 4-9). Progressive cell population is defined as cells moving with both $VAP > \text{PATH VELOCITY}$ ($25\mu/s$) and $STR > 80\%$ as determined by values defined for humans in the CASA ANALYSIS SETUP.

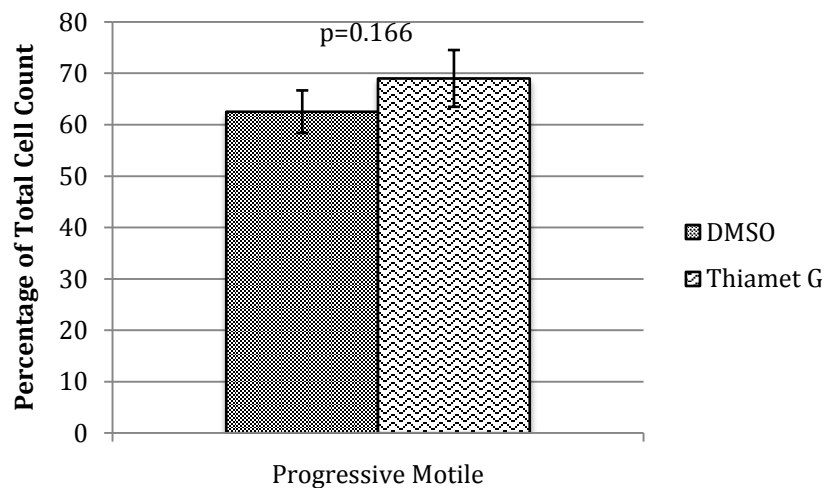


Figure 4-9: Influence of O-GlcNAcylation induction (Thiamet G) on human sperm progressive motility. The histogram shows that treatment with Thiamet G has no effects in the progressive cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

The percentage of progressive cells in the untreated population was 62.56% vs. 69.03% in the treated population. The p-value was 0.166, so no significant difference can be attributed to the result (figure 4-9).

On the next two charts I compare the VAP and VSL values (figure 4-10).

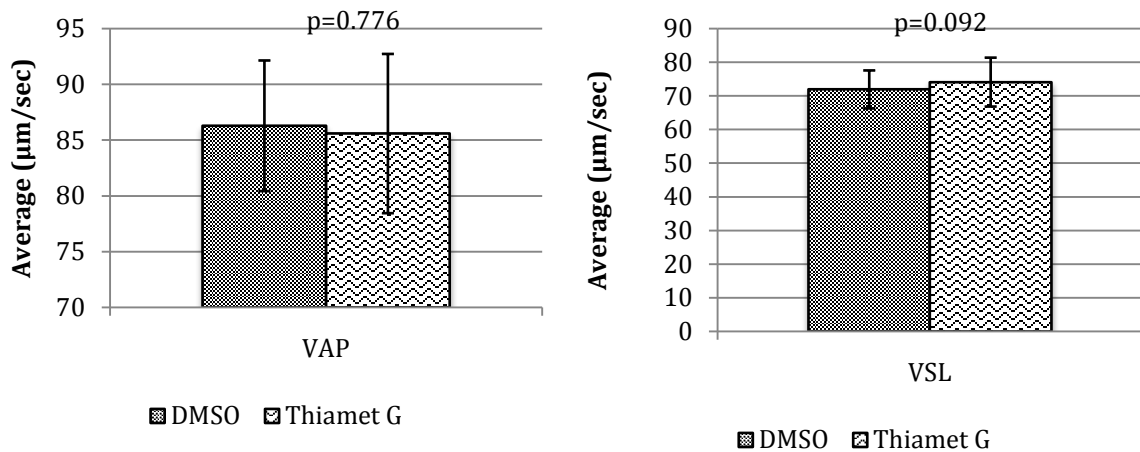


Figure 4-10: Influence of O-GlcNAcylation induction (Thiamet G treatment) on human sperm path velocity (VAP) and progressive velocity (VSL). The histogram shows that treatment with Thiamet G has no effects in VAP or VSL. Values are mean \pm SEM for 7 individual donor ejaculates

The average path velocity in untreated cells was 86.29 $\mu\text{m}/\text{sec}$ and in treated cells was 85.59 $\mu\text{m}/\text{sec}$. The p-value was 0.776, so treatment with Thiamet G does not affect the average path velocity of the cells. The average progressive velocity in untreated cells was 71.94 $\mu\text{m}/\text{sec}$ vs. 74.12 $\mu\text{m}/\text{sec}$ in treated cells. The p-value was 0.092, so no significant difference can be attributed to the difference in VSL (figure 4-10).

In the next chart, I compare the level of hyper-activated cells between treated and untreated populations (figure 4-11). Hyper-activated cells are cells defined as cells moving with both three parameters: $VCL \geq 150 \mu\text{m/s}$, $ALH \geq 7 \mu\text{m}$, $LIN \leq 50$.

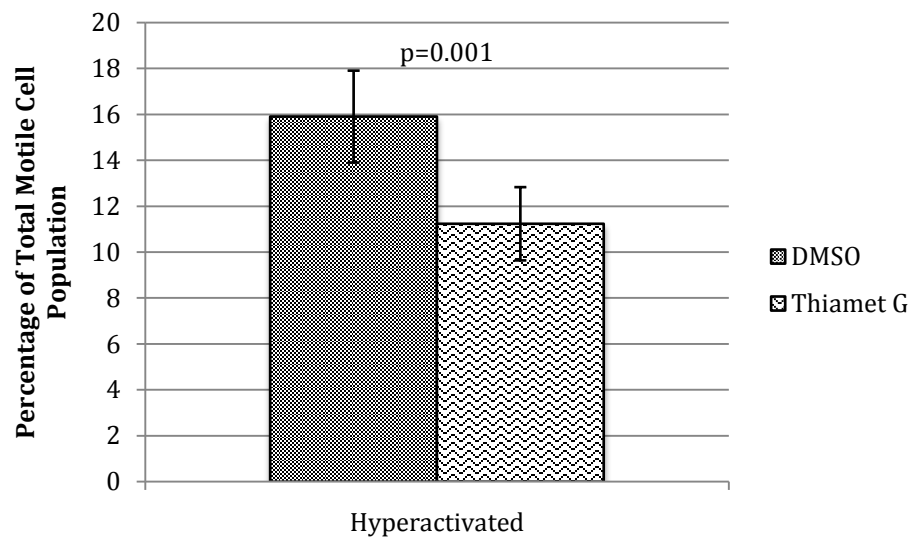


Figure 4-11: Influence of O-GlcNAcylation induction (Thiamet G) on human sperm hyperactivation levels. The histogram shows that treatment with Thiamet G significantly decreases the percentage of hyper-activated cells. Values are mean \pm SEM for 7 individual donor ejaculates.

In the un-treated population the percentage of hyper-activated cells was 15.91% vs. 11.24% in the treated population. So treatment with Thiamet G significantly reduces hyper-activation levels (figure 4-11).

In my attempt to investigate which of the parameters of the hyper-activated cells is affected the most, I compared the average values of VCL, LIN and ALH (figure 4-12).

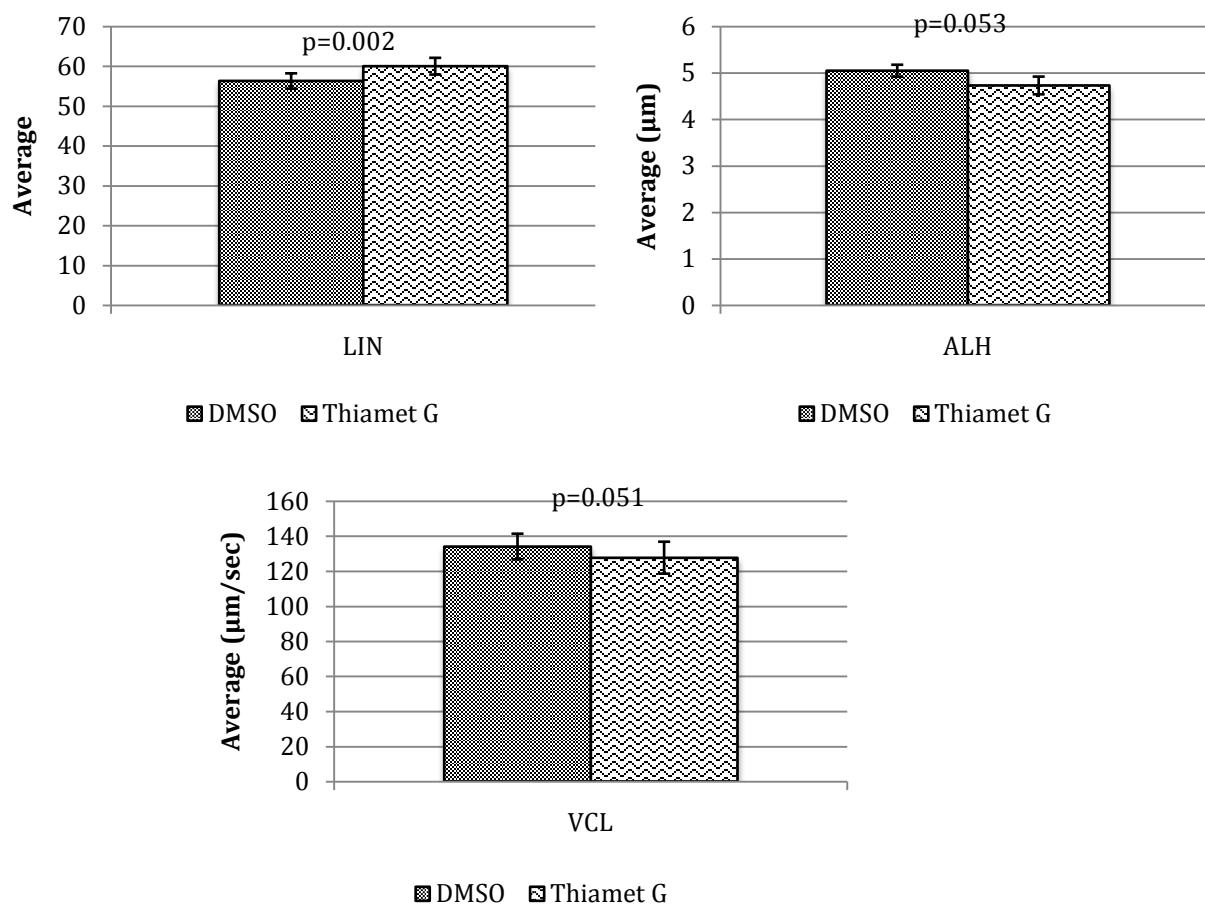


Figure 4-12: Influence of O-GlcNAcylation induction (Thiamet G treatment) on human sperm linearity levels (LIN), amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL). The histogram shows that treatment with Thiamet G significantly increases LIN but has no effects in ALH and VCL. Values are mean \pm SEM for 10 individual donor ejaculates.

Treatment with Thiamet G significantly increases only linearity out of the three parameters that contribute to hyperactivation. So the significant reduction of hyperactivation levels observed in figure 4-11, is the result of an increase in linearity.

4.7 Inhibitor 3: PUGNAc

PUGNAc is a potent in vitro inhibitor of the enzyme responsible for the removal of O-GlcNAc from proteins and can be used to increase O-GlcNAc levels on nuclear and cytoplasmic proteins in vivo. Studies have shown that PUGNAc approximately causes a 2-fold increase in O-GlcNAc levels in the human colon cancer cells, HT29, although the effects on individual proteins varied. O-GlcNAc levels in other cell lines tested (NIH 3T3, CV-1 and HeLa), were also affected by PUGNAc. At the concentration tested, PUGNAc is shown to be non-toxic for many cell lines (Haltiwanger et al., 1998, Park et al., 2005, Perreira et al., 2006, Shanmugasundaram et al., 2006).

The concentration used, was 100 μ M and the cells were incubated under capacitating conditions for 4 hours. At the end of the capacitating period, motility measurements were taken.

In the first chart (figure 4-13), I compare the static/motile cell population between treated and untreated cells. The static cells population consists of non-motile cells with size values defined by CASA. The CASA uses defined size values to avoid counting other cells types that can be found in semen, such as white or red blood cells.

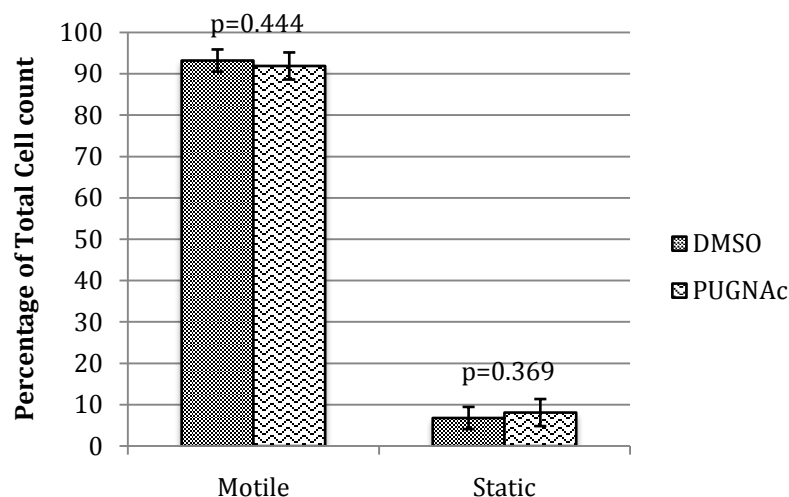


Figure 4-13: Influence of PUGNAc treatment on human sperm motility. The histogram shows that treatment with PUGNAc has no effects in the motile/static cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

The comparison of the static cell population between treated and untreated cells, provides the necessary information about the toxicity of the compound. Since there is no significant difference, no toxic effects can be attributed to the compound used to treat the cells (figure 4-13).

In order to be more specific, the percentage of the static cell population in untreated cells was 6.80% vs. 8.1% in the treated population. The p-value was 0.369, so no significant difference can be attributed to the result. On the other hand, the motile cell population in untreated cells was 93.20% vs. 91.89% in the treated population. The p-value was 0.444, so no significant difference can be attributed to the result (figure 4-13).

The motile cell population can be further divided into 3 categories (rapid, medium, slow). Slow cells are defined as cells with either of the following attributes: $VAP < VAP \text{ CUT-OFF} = 5 \mu/s$ or $VSL < VSL \text{ CUT-OFF} = 11 \mu/s$. Medium cells are moving with $VAP: \text{CUT-OFF} (5 \mu/s) < VAP < \text{PATH-VELOCITY} (25 \mu/s)$. Finally rapid cells are moving with $VAP > \text{PATH-VELOCITY} (25 \mu/s)$.

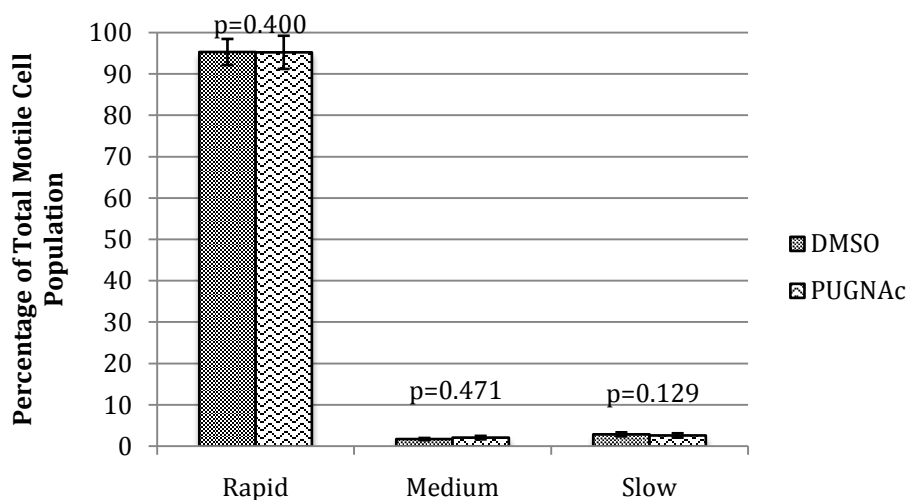


Figure 4-14: Influence of O-GlcNAcylation induction (PUGNac treatment) on the motile cell population. The histogram shows that treatment with PUGNac has no effects in the Rapid/medium/slow cell population.

Values are mean \pm SEM for 7 individual donor ejaculates.

No significant differences in all three cases were observed (figure 4-14).

The percentage of the rapid cell population in untreated cells was 95.32% vs. 95.23% in the treated population. The p-value was 0.400, so there is no significant difference. Next, the percentage of the medium cell population in untreated cells was 1.76% vs. 2.12% in the treated population. The p-value was 0.471, so no significant difference can be attributed to the result. Finally, the percentage of the slow cell population in untreated cells was 2.90% vs. 2.64% in the treated population. The p-value was 0.129, so no significant difference can be attributed to the result (figure 4-14).

The next chart compares the percentage of progressive cells between treated and untreated populations (figure 4.15). Progressive cell population is defined as cells moving with both VAP>PATH VELOCITY (25 μ /s) and STR>80% as determined by values defined for humans in the CASA ANALYSIS SETUP.

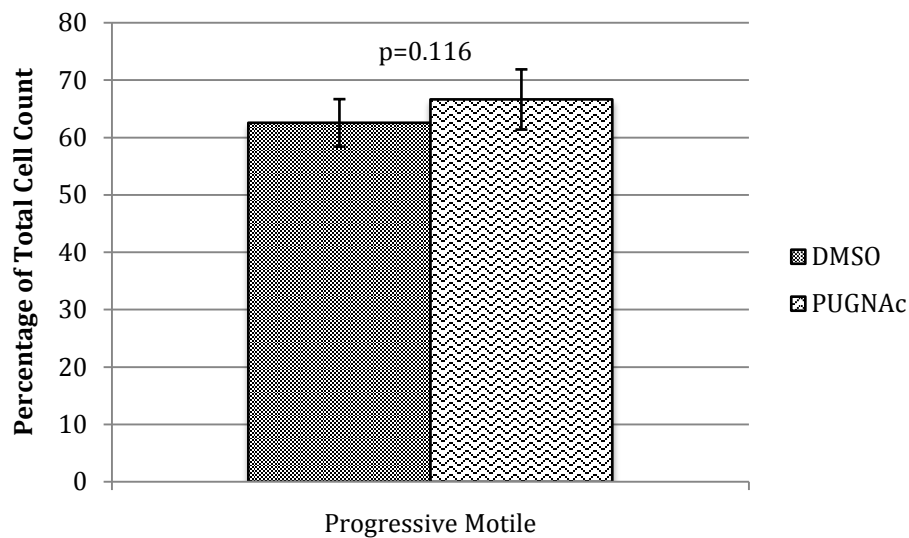


Figure 4-15: Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm progressive motility. The histogram shows that PUGNAc treatment has no effects in the progressive cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

The percentage of progressive cells in the untreated population was 62.56% vs. 69.03% in the treated population. The p-value was 0.166, so no significant difference can be attributed to the result (figure 4-15).

On the next two charts I compare the VAP and VSL values (figure 4-16).

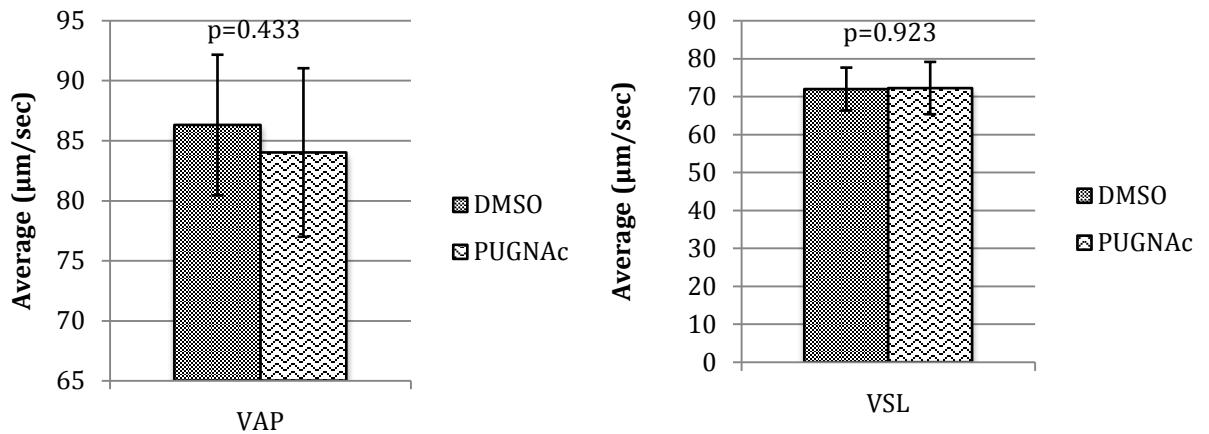


Figure 4-16: Influence of O-GlcNAcylation induction (PUGNac treatment) on human sperm path velocity (VAP) and progressive velocity (VSL). The histogram shows that PUGNac treatment has no effects in VAP or VSL. Values are mean $\pm\text{SEM}$ for 7 individual donor ejaculates

The average path velocity in untreated cells was 86.29 $\mu\text{m}/\text{sec}$ and in treated cells was 84.00 $\mu\text{m}/\text{sec}$. The p-value was 0.443, so treatment with PUGNac does not affect the average path velocity of the cells. The average progressive velocity in untreated cells was 71.94 $\mu\text{m}/\text{sec}$ vs. 72.20 $\mu\text{m}/\text{sec}$ in treated cells. The p-value was 0.923, so no significant difference can be attributed to the difference in VSL (figure 4-16).

In the next chart, I compare the level of hyper-activated cells between treated and untreated populations (figure 4-17). Hyper-activated cells are cells defined as cells moving with both three parameters: $VCL \geq 150 \mu\text{m/s}$, $ALH \geq 7 \mu\text{m}$, $LIN \leq 50$.

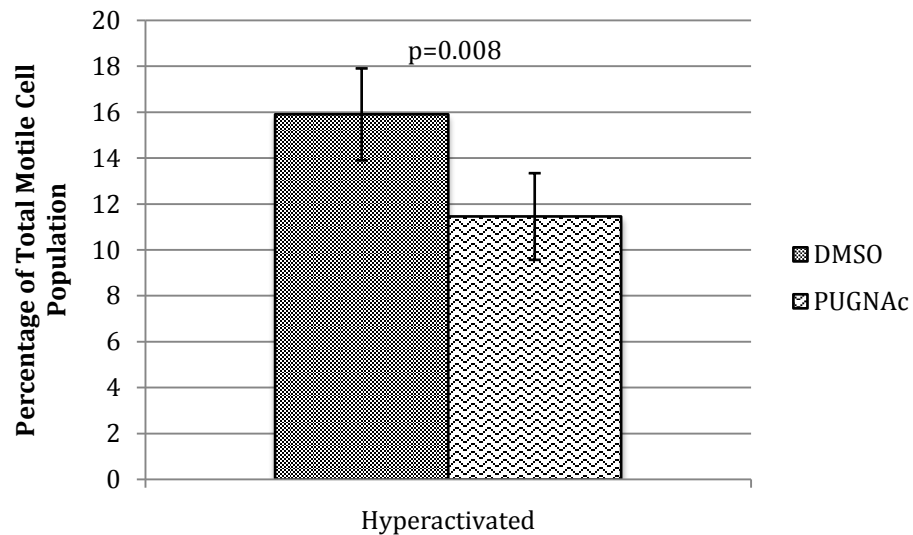


Figure 4-17: Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm hyperactivation levels. The histogram shows that treatment with PUGNAc significantly decreases hyper-activated cells. Values are mean \pm SEM for 7 individual donor ejaculates.

In the un-treated population the percentage of hyper-activated cells was 15.91% vs. 11.46% in the treated population. So treatment with PUGNAc significantly reduces hyper-activation levels.

In my attempt to investigate which of the parameters of the hyper-activated cells is affected the most, I compared the average values of VCL, LIN and ALH (figure 4-18).

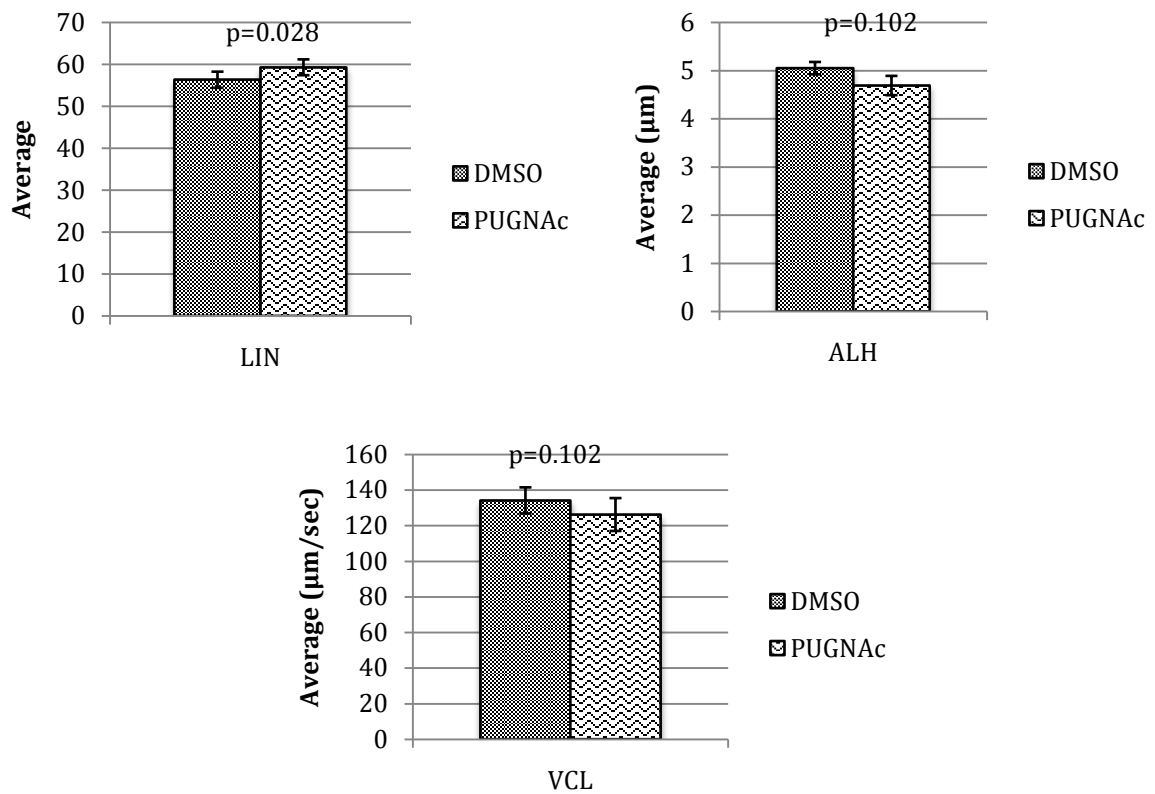


Figure 4-18: Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm linearity levels (LIN), amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL). The histograms show that PUGNAc treatment significantly increase LIN but has no effects in ALH and VCL. Values are mean \pm SEM for 10 individual donor ejaculates.

Treatment with PUGNAc significantly increases only linearity out of the three parameters that contribute to hyperactivation. So the significant reduction of hyperactivation levels observed in figure 4-17, is the result of an increase in linearity.

4.8 4Ac5S-GlcNAc

4Ac5S-GlcNAc is an inhibitor of the O-GlcNAc transferase (OGT) and its inhibition causes a dramatic reduction in O-GlcNAc levels.

The concentration used, was 10 μ M, and the cells were incubated under capacitating conditions for 4 hours. At the end of the capacitating period, motility measurements were taken.

In the first chart (figure 4-18), I compare the static/motile cell population between treated and untreated cells. The static cells population consists of non-motile cells with size values defined by CASA. The CASA uses defined size values to avoid counting other cells types that can be found in semen, such as white or red blood cells

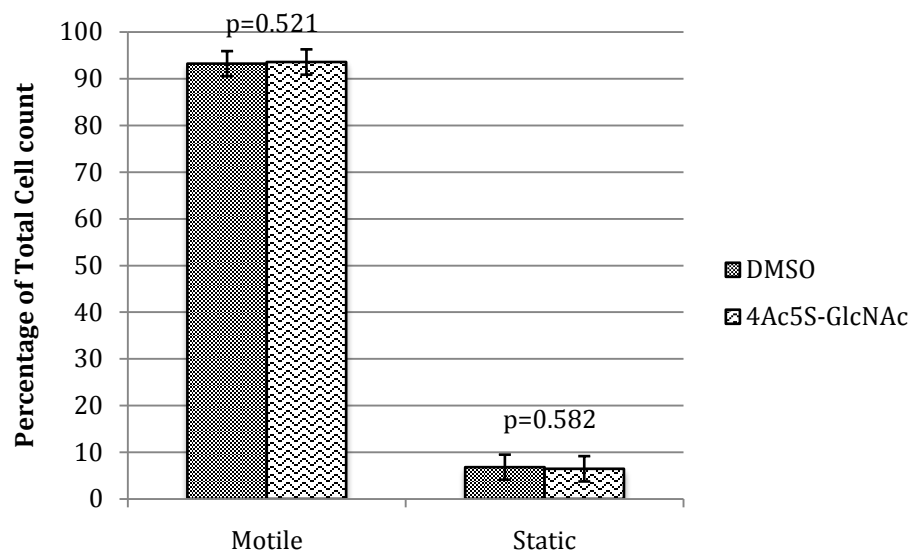


Figure 4-19: Influence of 4Ac5S-GlcNAc treatment on human sperm motility. The histogram shows that 4Ac5S-GlcNAc treatment has no effects in motile/static cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

The comparison of the static cell population between treated and untreated cells provides the necessary information about the toxicity of the compound. Since there is no significant difference, no toxic effects can be attributed to the compound used to treat the cells (figure 4-18).

In order to be more specific, the percentage of the static cell population in untreated cells was 6.80% vs. 6.46% in the treated population. The p-value was 0.582, so no significant difference can be attributed to the result. On the other hand, the motile cell population in untreated cells was 93.20% vs. 93.54% in the treated population. The p-value was 0.582, so no significant difference can be attributed to the result (figure 4-18).

The motile cell population can be further divided into 3 categories (rapid, medium, slow). Slow cells are defined as cells with either of the following attributes: $VAP < VAP \text{ CUT-OFF} = 5 \mu/s$ or $VSL < VSL \text{ CUT-OFF} = 11 \mu/s$. Medium cells are moving with $VAP \text{ CUT-OFF} (5 \mu/s) < VAP < \text{PATH-VELOCITY} (25 \mu/s)$. Finally rapid cells are moving with $VAP > \text{PATH-VELOCITY} (25 \mu/s)$.

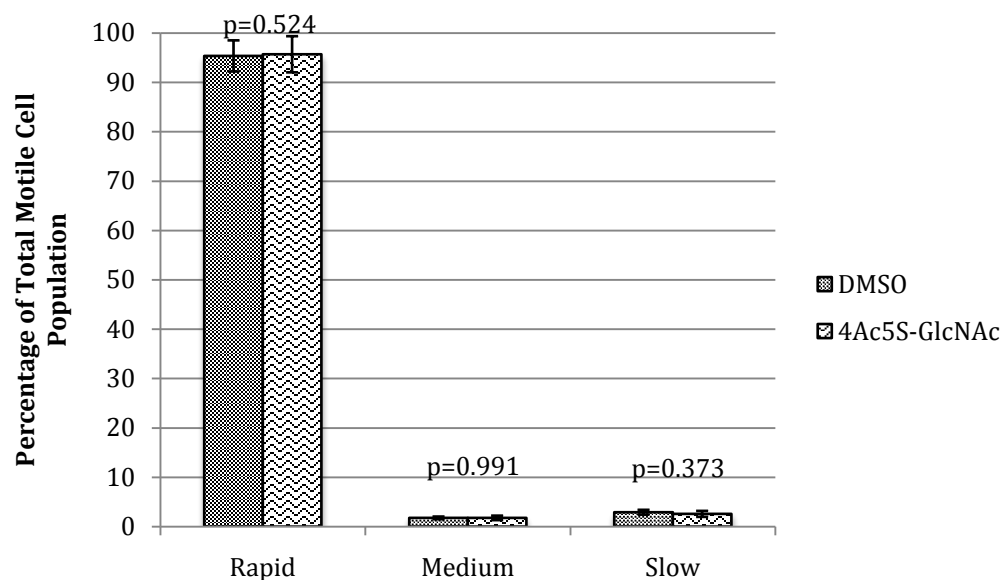


Figure 4-20: Influence of 4Ac5S-GlcNAc treatment on the motile cell population. The histogram shows that 4Ac5S-GlcNAc treatment doesn't affect Rapid/medium/slow population. Values are mean \pm SEM for 7 individual donor ejaculates.

No significant differences in all three cases were observed (figure 4-19).

The percentage of the rapid cell population in untreated cells was 95.32% vs. 95.65% in the treated population. The p-value was 0.524, so there is no significant difference. Next, the percentage of the medium cell population in untreated cells was 1.766% vs. 1.764% in the treated population. The p-value was 0.991, so no significant difference can be attributed to the result. Finally, the percentage of the slow cell population in untreated cells was 2.90% vs. 2.57% in the treated population. The p-value was 0.373, so no significant difference can be attributed to the result (figure 4-19).

The next chart compares the percentage of progressive cells between treated and untreated populations (figure 20). Progressive cell population is defined as cells moving with both VAP>PATH VELOCITY (25 μ /s) and STR>80% as determined by values defined for humans in the CASA ANALYSIS SETUP.

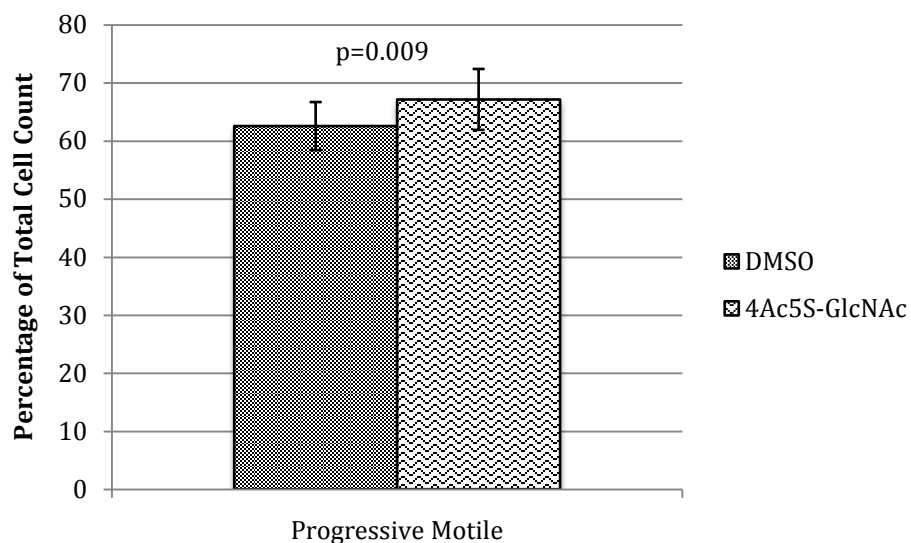


Figure 4-21: Influence of 4Ac5S-GlcNAc treatment on human sperm progressive motility. The histogram shows that 4Ac5S-GlcNAc treatment, has no effects in the progressive cell population. Values are mean \pm SEM for 7 individual donor ejaculates.

The percentage of progressive cells in the untreated population was 62.56% vs. 67.15% in the treated population. The p-value was 0.009, so no significant difference can be attributed to the result (figure 4.20).

On the next two charts I compare the VAP and VSL values.

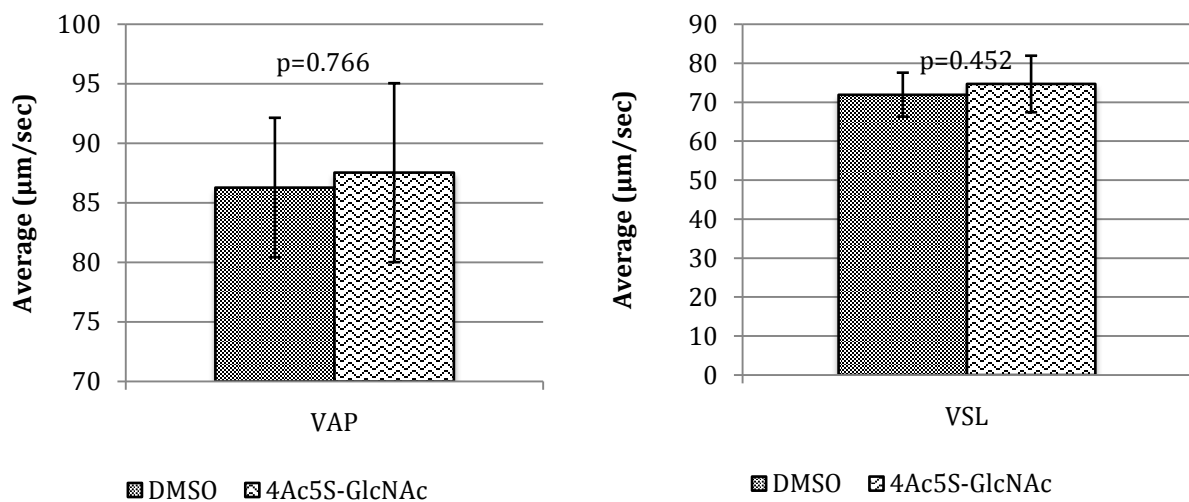


Figure 4-22: Influence of O-GlcNAcylation induction (4Ac5S-GlcNAc treatment) on human sperm path velocity (VAP) and progressive velocity (VSL). The histograms show that 4Ac5S-GlcNAc treatment has no effects in VAP or VSL. Values are mean \pm SEM for 7 individual donor ejaculates

The average path velocity in untreated cells was 86.29 $\mu\text{m}/\text{sec}$ and in treated cells was 87.54 $\mu\text{m}/\text{sec}$. The p-value was $0.766 > 0.05$, so treatment with 4Ac5SGlcNAc does not affect the average path velocity of the cells. The average progressive velocity in untreated cells was 71.94 $\mu\text{m}/\text{sec}$ vs. 74.68 $\mu\text{m}/\text{sec}$ in treated cells. The p-value was 0.452, so no significant difference can be attributed to the difference in VSL (figure 4.21).

In the next chart (figure 4.22), I compare the level of hyper-activated cells between treated and un-treated populations (figure 4-8). Hyper-activated cells are cells defined as cells moving with both three parameters: $VCL \geq 150 \mu\text{m/s}$, $ALH \geq 7 \mu\text{m}$, $LIN \leq 50$.

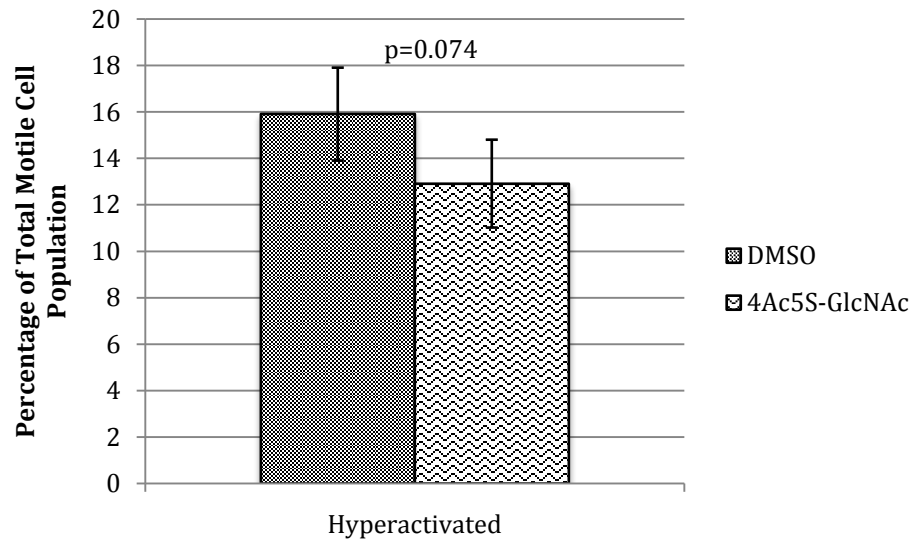


Figure 4-23: Influence of 4Ac5S-GlcNAc treatment on human sperm hyperactivation levels. The histograms show that 4Ac5S-GlcNAc treatment has no effects in the percentage of hyper-activated cells. Values are mean \pm SEM for 7 individual donor ejaculates.

In the un-treated population the percentage of hyper-activated cells was 15.91% vs. 12.92 % in the treated population. So treatment with 4Ac5SGlcNAc significantly reduces hyper-activation levels.

In my attempt to investigate which of the parameters of the hyper-activated cells is affected the most, I compared the average values of VCL, LIN and ALH.

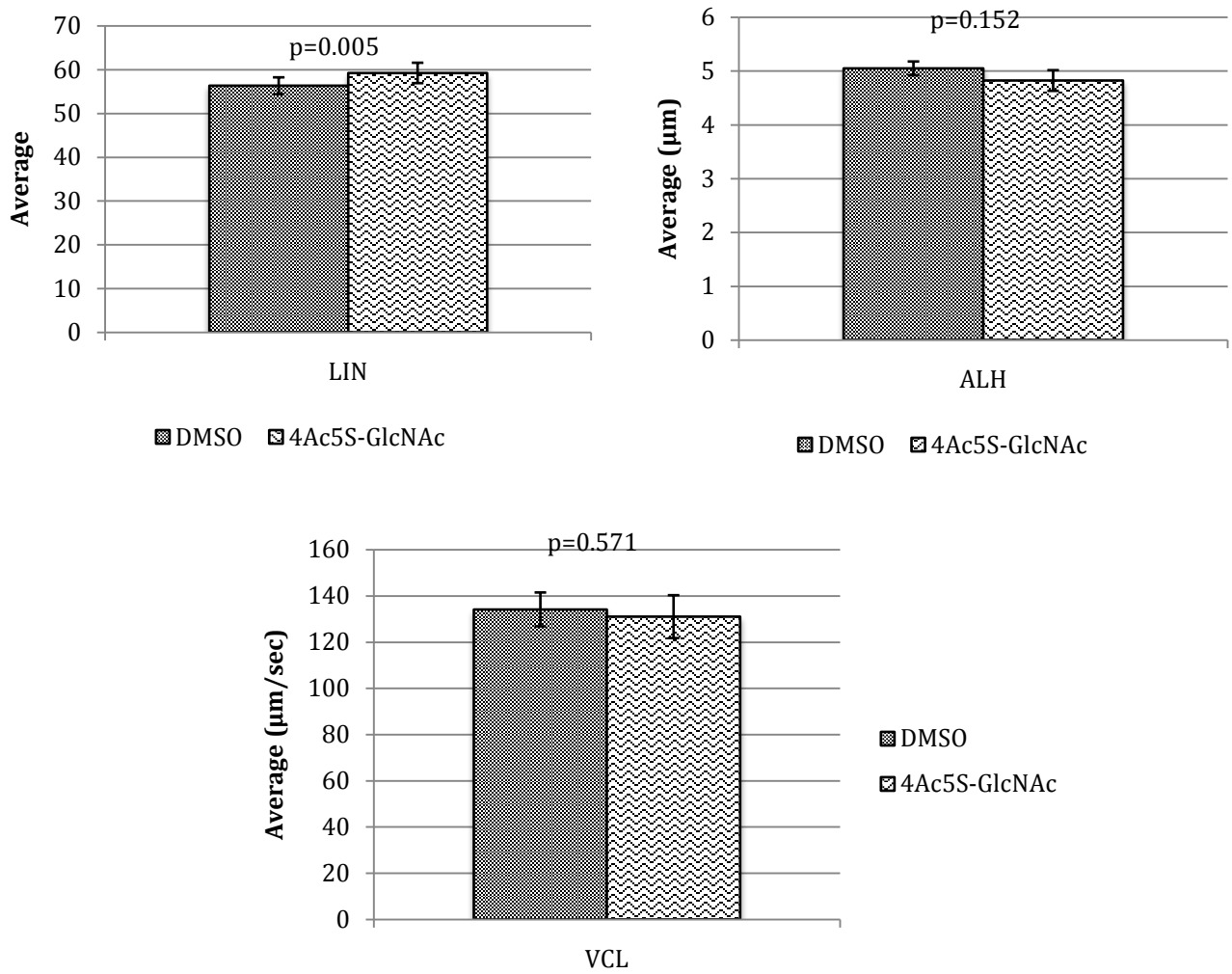


Figure 4-24: Influence of O-GlcNAcylation induction (4Ac5S-GlcNAc treatment) on human sperm linearity levels (LIN), amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL). The histogram shows that treatment with 4Ac5S-GlcNAc significantly increases LIN, but has no effects in ALH and VCL. Values are mean \pm SEM for 10 individual donor ejaculates.

4.8 Protein Results

The aim of this part of the project is to detect changes in O-GlcNAcylation levels at a protein level caused by the inhibitors. From our motility results became apparent that GNS is the most potent inducer of O-GlcNAcylation, as significantly affected four sperm motility parameters (VAP, VCL, Hyper-activation, ALH). Four individual donor ejaculates were incubated under capacitating conditions with DMSO (vehicle control) and GNS (inhibitor). After a four-hour capacitation period, protein was extracted and analysed by Western blotting. One out of four donors expressed an increase in O-GlcNAcylation levels (Donor 6), which was accompanied with an increase in tyrosine phosphorylation levels. This fact suggests that capacitation was normally progressing. One donor (Donor 4) expressed a small increase in O-GlcNAcylation levels. The tyrosine-phosphorylation for the same donor was slightly increased. Finally the O-GlcNAcylation levels in the rest of the donors remained unchanged (figure 4-25).

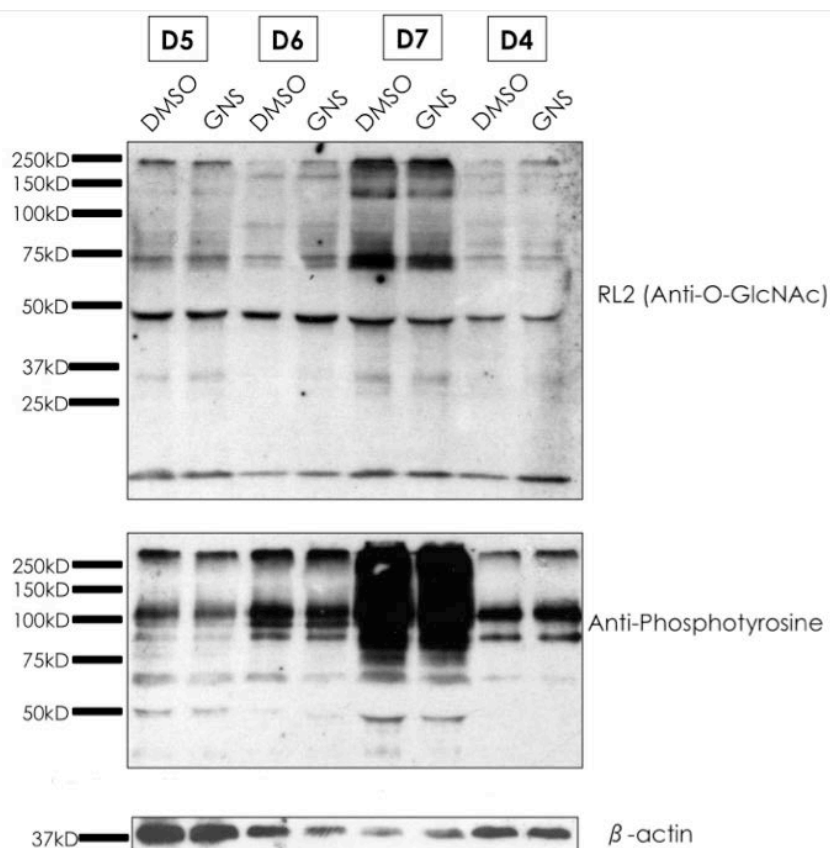


Figure 4-25: Influence of O-GlcNAcylation induction (GNS treatment) on human sperm protein O-GlcNAcylation levels on 4 different donors.

In the next experiment (figure 4-26) one donor ejaculate was incubated under non-capacitating conditions (NCB), capacitating conditions with the vehicle control (DMSO) and capacitating conditions with the three inhibitors (GNS, Thiamet G, PUGNAC). Our results reveal that incubation under capacitating conditions without inhibitors (STF) leads to a decrease in O-GlcNAcylation levels. This results come to an agreement with the results in Chapter 3. At the same time an increase in tyrosine phosphorylation levels was observed, fact that suggests capacitation is progressing normally. Capacitation with the three inhibitors, causes in every case an increase in O-GlcNAcylation levels. Thiamet G appears to be the most potent inhibitor.

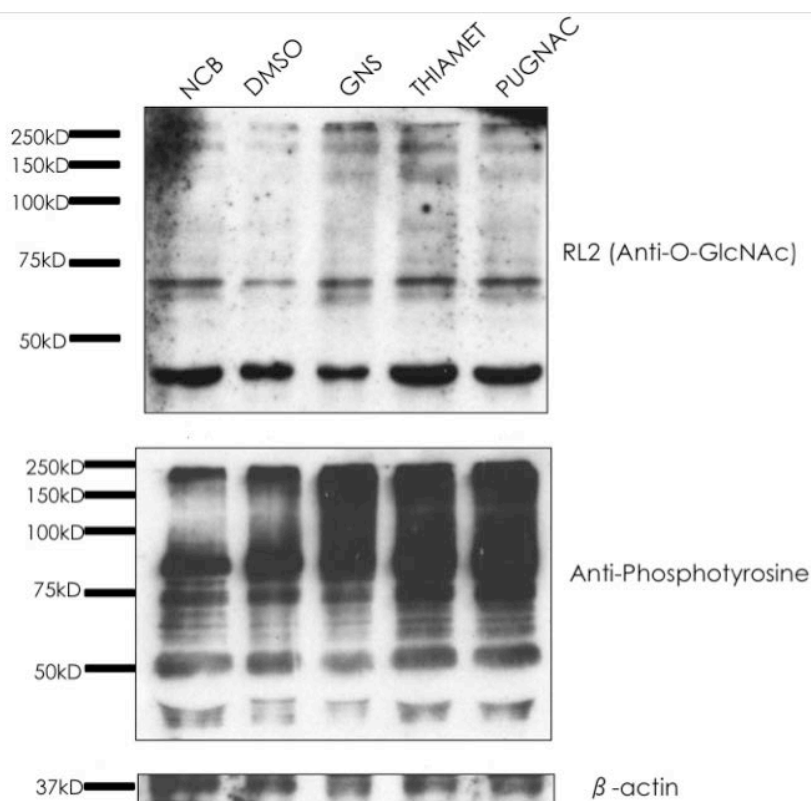


Figure 4-26: Influence of O-GlcNAcylation induction (3 different inhibitors) on human sperm protein O-GlcNAcylation levels. The results indicate that O-GlcNAcylation induction with GNS/Thiamet G or PUGNAC significantly increases the concentration of O-GlcNAcylated proteins.

4.9 Discussion

In this part of the project, the effects of 4 different inhibitors in sperm motility were studied. The inhibitors are: GNS, Thiamet G, PUGNAc and 4Ac5S-GlcNAc. The first 3 inhibitors block OGA, the enzyme responsible for the removal of O-GlcNAc, so the cells were submitted under conditions, which induce O-GlcNAcylation. 4Ac5S-GlcNAc blocks OGT, the enzyme responsible for the addition of O-GlcNAc to ser/thr residues, so it blocks O-GlcNAcylation in the cells. The results show that GNS, Thiamet G and PUGNAc significantly cause impaired hyper-activation. 4Ac5S-GlcNAc treatments had no significant effects in hyperactivation levels. This can be explained by the fact that the cells were treated with relatively low concentration of the drug. Maybe gradual increase of the drug concentration will give significant results. Finally 4Ac5S-GlcNAc is a newly developed drug and little is known about its potency and its effect in sperm cells.

Chapter 5: General discussion

It was 40 years ago, when Yanagimachi observed that hamster spermatozoa swimming in the oviduct exhibited a vigorous motility pattern which he named hyperactivation (Yanagimachi, 1970). Since then, hyperactivation has been the centre of vast amount of studies trying to extensively understand the physiological factors leading to hyperactivation, as acquisition of the hyperactivated state by the sperm is necessary for successful in vivo fertilization. Factors that inhibit the transition of sperm to a hyperactivated state are all related with infertility.

Male factor infertility accounts for 35% (de Kretser, 1997) of all infertility cases worldwide and can be caused by various factors, including hormonal disorders, infections and anatomical disorders (Mosher and Pratt, 1991, de Kretser et al., 1998a). It is often associated with impaired sperm motility and morphology for which there is no specific therapeutic treatment. Currently, the only treatment available, for sub fertile men, are Assisted Reproduction Techniques (ART). It has come to light that the modification and expression of human sperm proteins play a crucial role in sperm function. The present study was designed to explore the molecular nature of sperm capacitation in order to find suitable treatment options for men suffering from infertility. This includes the identification of all post-translational modifications on sperm proteins, which can serve as potential targets for the development of biomarkers and the use of drugs to cure sperm dysfunction.

Little is known about the mechanisms responsible for the transition of sperm into the hyper-activated state. Current evidence suggests that cAMP/PKA and Ca^{2+} signalling pathway are of paramount importance for the regulation of sperm motility (Suarez et al., 1987, Lindemann and Goltz, 1988, Yanagimachi, 1994, Ho and Suarez, 2003). Also, other pathways have been characterized, such as G-protein mediated pathways implicated in sperm motility. Although, as we discussed, many factors are involved in

the acquisition of the hyper-activated state, in this study only the role of post-translational modifications was my main area of study.

Post-translational modifications play a very important role from the early stages of spermatogenesis to epididymal maturation and finally capacitation and acquisition of the capacity to fertilize. My aim was to identify if O-GlcNAcylation is present in sperm proteins and how is involved in the process of capacitation.

In the first part of the study, several O-GlcNAc proteins were identified. Un-capacitated sperm was shown to exhibit a higher level of O-GlcNAcylation compared with sperm that were incubated in capacitating media. Finally, the level of O-GlcNAcylation appeared to decrease markedly following capacitation.

Interpretation of western blot results was a vital part of my project. Only by being aware of the limitations of the procedure, it is possible to correctly interpret the results. Western blotting is one of the most common procedures in biochemical labs. It separates proteins from a sample by size, then tests using antibodies to determine whether a given protein is present. Despite its popularity, Western blotting has several disadvantages. Western blots are non-quantitative. The results reveal, if a particular protein is present, but they do not make it possible to accurately quantify, how much of the protein is present. Moreover, the Molecular weight of a protein can only be estimated with western blotting, rather than determined precisely. Finally antibodies sometimes exhibit some off-target binding, which can make for poorer results.

In the second part of the study, I used specific, highly potent inhibitors to induce O-GlcNAcylation and examine its effects in the motility of spermatozoa.

The activity of the O-GlcNAcase (OGA) was disrupted using the selective inhibitor, GlcNAcstatin. Currently, the GlcNAcstatins are the most potent OGA inhibitors available (Dorfmueller et al., 2009). They bind directly to the active site of OGA by hydrogen bonding, and induce global increases in O-GlcNAc levels. GlcNAcstatins

show a 100.000 times enhanced selectivity for O-GlcNAcase over human lysosomal hexosaminidases, an established concern over the commonly used O-GlcNAcase inhibitor, PUGNAc, making them better tool for reasearching the effects of induction of O-GlcNAcylation. As additional verification, Thiamet G, an OGA inhibitor, structurally unrelated to GlcNAcstatin C was also used, to further test OGA inhibition.

From the motility results became apparent that induction of O-GlcNAcylation significantly affects at least four sperm motility parameters, one of which is hyper-activation.

In order to understand the basis of the effects of O-GlcNAcylation, we have to come to a better understanding of capacitation and the core enzymes, which control it. Capacitation is a combination of early and late events that has been associated with a list of endpoints. Several pathways have been characterized, but the molecular basis of capacitation remains elusive. The main pathway starts from an increase in intracellular bicarbonate. The movement of the bicarbonate inside the cell induces an increase in intracellular pH, which stimulates soluble Adenylyl Cyclase. The rise of intracellular cAMP activates Protein Kinase A, which phosphorylates various proteins, causing the initiation of several signaling pathways. PKA refers to a family of ser/thr kinases whose activity is dependent on cellular levels of cAMP. Blocking PKA with specific inhibitors blocks the whole process of phosphorylation. So PKA is one of the core enzymes that control capacitation. Is it possible that O-GlcNAcylation is involved in the regulation of PKA?

Francisco et al. demonstrated that induction of O-GlcNAc levels regulates the phosphorylation of some PKA substrates. More specifically, Western analysis in primary chicken forebrain neurons, revealed that the effect of increasing cAMP levels on the phosphorylation of PKA substrates was antagonized by increasing O-GlcNAc levels (Francisco et al., 2009). So their results provide a functional correlate to a

previous study demonstrating reciprocal relationship between O-GlcNAcylation and phosphorylation on neuronal cytoskeletal proteins downstream of cAMP-PKA signaling (Griffith and Schmitz, 1999). In terms of sperm related content, induction of O-GlcNAcylation possibly blocks or inhibits the phosphorylation of “some” PKA substrates, causing significantly impaired hyperactivation. I mention “some” substrates because on the one hand, one PTM antagonizes the other and on the other hand Tyrosine phosphorylation is increased during induction of O-GlcNAcylation and it is known that Tyrosine phosphorylation increase is a result of a protein kinase cascade downstream of PKA.

Another way that possibly O-GlcNAc effects sperm capacitation is through the proteasome itself. As I discussed in chapter 1.9.7, O-GlcNAc is involved by two different ways in the degradation process. First by altering the targeting of some proteins to the proteasome and secondly by altering the activity of the proteasome itself. Kong et al. demonstrated that proteasome plays a huge role in mammalian capacitation (Kong et al., 2009). By incubating highly motile sperm with proteasome inhibitors they inhibited capacitation and they also changed the pattern of tyrosine phosphorylation. This fact suggests the importance of the proteasome in regulating protein phosphorylation patterns during capacitation by possibly by degrading specific kinases that are not needed. Since O-GlcNAc regulates the proteasome itself, it is possible that O-GlcNAcylation possibly regulates the presence or absence of specific kinases during the capacitation process through the O-GlcNAcylation status of the proteasome.

More work needs to be done, in order to completely elucidate the signalling pathways responsible for the induction of sperm hyper-activation. Hopefully we will get better, in the understanding of the nature of this complex biochemical process that renders spermatozoa able to fertilize. The better understanding will allow us to develop drugs to

treat male infertility and diminish the need of consistently resorting to ART, which are expensive and invasive.

Appendix

1 Role of O-GlcNAc modification in embryonic stem cells

Mouse embryonic Stem Cells (MESC)s are derived from the inner cell mass of the blastocyst and have the ability to self-renew and differentiate into all types of 3 embryonic germ layers (endoderm, mesoderm, ectoderm). These 3 germ layers give rise to more than 220 cell types in the adult body including germ cells. The understanding of their function will allow us to alter the way people are being treated. Their plasticity and their unlimited capacity for self-renewal promote them as perfect candidates for cell replacement therapies and especially treatment of male infertility. Male factor infertility is identified in almost 50% of infertile couples while it is the sole cause in 20-30% of infertile couples. However, the signalling pathways that control cell-fate are vast and hugely complex. Inside the cell numerous post-translational modifications take place, but the discovery of O-GlcNAc on cytoplasmic proteins attracted our interest. Therefore this part of my project is about the investigation of O-linked Acetylglucosaminated proteins in several of the key signalling pathways implicated in stem cell proliferation, cell viability and Self Renewal.

1.1 Derivation of human and mouse ES cells

The majority of human embryos come from the surplus of embryos that In Vitro Fertilization (IVF) generates. During this process multiple embryos are produced. The most suitable according to morphological criteria are kept for implantation into the patient and the rest of them can be donated for research with the absolute consent of the parents. The embryos are cultured until they reach the blastocyst stage. For human this period is 4-5 days post fertilization, at which time the human blastocyst consists of 50-150 cells. The inner cell mass (ICM) is separated from the trophoectoderm, which is responsible for the formation of extra-embryonic tissue, with Immunosurgery. In Immunosurgery the cells of trophoectoderm are destroyed by brief exposure to

antibodies directed against human cell in tandem with complement activity (Chen and Melton, 2007). The remaining inner cell mass is plated onto cells that will supply support. Expansion of the inner cell mass will lead to a formation of an embryonic cell line. The cells must be separated every 4-7 days and they can retain their pluripotency even after months of proliferation. In mice, the ES cells are derived from the inner cell mass of the embryo, which is harvested from the donor-mother animal by following similar techniques.

1.2 Self-renewal and signalling pathways controlling self-renewal

Self-renewal is the ability of the cell to renew itself indefinitely, while maintaining the undifferentiated state. This process requires cell cycle control and involves complex networks. These networks are regulated by cell extrinsic signals from the microenvironment of the stem cell. We try to elucidate the mechanisms that are responsible for this process.

1.3 The LIF Pathway

The first mouse embryonic stem cells were cultured on mouse embryonic fibroblasts (MEF). The need for this feeder layer was negated by the addition of conditioned media from buffalo rat liver cells (BRLs) directly to mESC cultures. The media contained an IL-6 family cytokine that inhibits ES cell differentiation. After its secretion LIF binds to a heteromeric complex, which consists of gp130 and LIF receptor, and activates Janus Kinase (JAK). JAK phosphorylates STAT proteins (STAT1 and STAT3) and activates them. STAT3 relocates to the nucleus as a homodimer and activates c-Myc, which is a transcription factor responsible for cell self-renewal. LIF by itself cannot promote self-renewal but it requires the presence of BMP-4 or serum. Secreted BMP-4 binds to a tetra-dimeric complex that forms BMP-4 membrane receptor and triggers the phosphorylation of R-Smad (Receptor Regulated Smad (R-Smad: Smad1,5 and 8) proteins. Activated R-Smad proteins form a complex with co-Smad (Smad4), and the

complex translocate into the nucleus to regulate target gene expression (Datto and Wang, 2000). They induce the expression of inhibitor of differentiation (Id). Id protein has a very important role in cell fate determination. If Id protein is overexpressed in mouse ES cells, LIF is able to maintain self-renewal of ES cells even with the absence of BMP-4 (Ying et al., 2003, Fei and Chen, 2010). BMP has been also reported, to be implicated in several lineage commitment pathways (Seuntjens et al., 2009).

LIF through its receptor gp130 has the ability to activate ERK (Niwa et al., 1998, Burdon et al., 1999). Activation of the BMP pathway causes the induction of dual-specificity phosphatase 9 (DUSP9). DUSP9 is an ERK-specific phosphatase. So BMP helps mouse ESCs maintain their self-renewal state by inhibiting the stimulating effect of LIF and FGF4 signalling on ERK activity. The mechanistic background behind the inhibitory effect consists of a Smad-dependent up-regulation of DUSP9. It is also proposed that DUSP9 can substitute BMP4 to sustain ESC Self-Renewal when combined with LIF (Li et al., 2012).

1.4 Other factors that induce self-renewal

Nichols showed that LIF receptor gp130 ^{-/-} mouse can develop to a post the blastocyst stage, suggesting that additional factors may also be involved in maintaining pluripotent cells in vivo (Nichols et al., 2001). In 2003 a new homeobox transcription factor (Nanog) was discovered which is specifically expressed in ES cells. Mouse Nanog protein consists of 280 amino acids and contains a homeobox domain (Homeobox genes encode transcription factors that typically switch on cascades of other genes). It consists of three domains. A C-terminal, a N-terminal and a homeodomain but only C-terminal exhibits high trans-activator function, suggesting that only C-terminal domain is functionally dominant. C-terminal also has a highly conserved 10 pentapeptide repeat (WR). Nanog has the ability to promote stem cell self-renewal and pluripotency. Deletion of the Nanog gene results in loss of pluripotency in both ICM (Inner Cell

Mass) and ES cells (Mitsui et al., 2003). The above distinct abilities come as a result of a LIF-independent transcription factor network, as overexpression of Nanog does not lead to the activation of STAT3. A lot of evidence indicates that Nanog functions through an independent pathway of Stat3. CAG-elevated ES cells show no increase in phosphorylation kinetics of Stat3. Secondly inactivation of Stat 3 pathway via LIF depletion leads to the formation of a different phenotype in comparison with Nanog null cells. Finally addition of LIF to CAG-Nanog cell culture enhances growth of ES cells [50]. Also addition of Janus-kinase inhibitors does not affect Nanog levels.

Nanog is not acting alone to maintain pluripotency. Along with other key regulatory pluripotent factors such as Oct4 and Sox2, form a regulatory network to support or limit each other's expression level. Nanog and Oct4 are homeodomain proteins and Sox2 is a High-Mobility Group factor (High-Mobility Group or HMG proteins are chromosomal proteins with main aim to help with transcription, replication, recombination and DNA repair). Oct4/ Sox2 bind to the Nanog promoter in vitro and in vivo and promote Nanog transcription in pluripotent cells (Rodda et al., 2005). However Oct4 deficient mice embryos express Nanog suggesting that also other pluripotency factors contribute to the regulation of Nanog expression. Reporter assays reveal that FoxD3, a forkhead family transcription factor, can activate the Nanog promoter. Chromatin immuno-precipitation (ChIP) assays confirm the ability of FoxD3 to bind Nanog promoter region in vivo (Pan et al., 2006). Except FoxD3 recent evidence shows that also the tumour suppressor gene, p53, can bind to the Nanog promoter in vivo and in vitro. Finally treatment of p53 ^{-/-} ES cells with retinoic acid (RA) during the differentiation still keeps Nanog down regulated, fact that suggests that other negative regulator factors are involved in suppressing Nanog during ES cell differentiation (Lin et al. (2005).

1.5 Canonical Wnt signalling in ESC self-renewal

B-Catenin is a protein that in humans is encoded by the CTNNB1 gene, and is the core component of the canonical Wnt pathway. When the Wnt ligands are absent, β -catenin gets phosphorylated by GSK3 and subsequently degraded by proteolysis. When the Wnt ligands are present, they bind with β -catenin, preventing its degradation. Accumulated hypo-phosphorylated β -catenin enters nucleus causing the up regulating of Stat3 mRNA.

1.6 Signalling pathways controlling differentiation

Fibroblast Growth Factors (FGFs) and their receptors (tyrosine kinases) play an important role in regulating pluripotency and lineage segregation in pluripotent mammalian stem cells. Binding of the ligand to the receptor results in the auto-phosphorylation of tyrosine residues in the intracellular region of an FGF receptor (FGFR). The signal is further relayed through four distinct pathways: the Janus kinase/signal transducer and activator of transcription (Jak/Stat), phospho-inositide phospholipase C (PLC γ), phosphatidylinositol 3- kinase (PI3K) and Erk pathways.

5.1.7 Therapeutic potential in male infertility

One day, men who lose the ability to produce sperm after chemotherapy may be able to regain their fertility because of stem cells. Men possess spermatogonial stem cells, that have the ability to produce spermatozoa and can be frozen before any exposure of the patient to therapeutic agents. A few months after the cancer treatment the stem cells can be re-injected back to patient testis and re-establish sperm production.

2 Materials and Methods

2.1 Materials

1. Proliferation Media Components:

- GMEM (Glasgow Modified Eagles Media)(500ml)(Life Technologies):
It contains a combination of amino acids, salts (calcium chloride, potassium chloride, magnesium sulphate, sodium chloride and monosodium phosphate), glucose and vitamins (folic acid, nicotinamide, riboflavin, B₁₂). It also contains iron and phenol red. Phenol red is used as pH indicator, which helps in monitoring the changes of the pH in the cell culture. When the pH becomes acidic (below 6.8), then the colour of the media changes from red to yellow.
- Fetal Bovine Serum (FBS)(50ml): Induction of coagulation is followed by conversion of fibrinogen to fibrin, which leads to the formation of a blood clot. The portion of plasma remaining after coagulation is the FBS. FBS contains a rich variety of proteins and growth factors, which support the proliferation of the cells.
- Sodium Pyruvate 100x (5ml)(GIBCO-Invitrogen): Sodium pyruvate is added to cell culture because it is an anti-oxidant, so it provides an effective layer of protection against hydrogen peroxide (Giandomenico et al., 1997) but it is also an additional source of energy along with the utilisation of glucose because it participates in the production of the high energy intermediate ATP within the cells.
- MEM Non-Essential Amino Acids (NEAA) (5ml) (GIBCO-Invitrogen): Addition of Non-Essential Amino Acids to medium improves growth and viability of propagating cell in the culture.

- Mercaptoethanol (3,5 μ L): is a potent reducing agent used in cell culture medium to prevent toxic levels of oxygen radicals.
- Leukaemia Inhibitory Factor (LIF) (1/1000): LIF is an interleukin 6 family cytokine. Addition of LIF in to the culture media inhibits ES cell differentiation by STAT3 pathway.

2. Differentiation Media (N2B27):

- Neurobasal Media (250ml) (Invitrogen)
- DMEM-F12 (250ml) (Invitrogen)
- N2 supplement (2.5ml): N2 is a serum-free supplement and is suitable for the growth, survival and expression of post-mitotic neurons.
- B27 supplement (5ml): B27 is a serum-free supplement that is used along with Neurobasal and supports the growth and viability of neurons.
- Glutamine (5ml)
- Mercaptoethanol (3.5 μ l)

3. Dulbecco's Phosphate Buffered Saline (dPBS): It's a isotonic buffer system intended to provide maintenance of the pH of the cell culture in to the physiological range of 7.2-7.6 and at the same time provides cells with water and certain bulk of inorganic ions which are essential for normal cell metabolism. When it is combined with carbohydrates, such as glucose, which serve as energy sources, dPBS can sustain the viability of the cells for a limited period of time.

4. DMSO (1mM): vehicle control

5. GlcNAcstatin (1 μ M): GlcNAcstatin binds directly to the active site of OGA by hydrogen bonding and blocks it. The above reaction results in increased O-GlcNAc levels.

6. GalNacstatin (1 μ M): GalNacstatin is an inactive isomer of GlcNAcstatin. It does not inhibit OGA but is structurally similar to GlcNAcstatin. This means any effects seen in GlcNAcstatin treatment can be more confidently attributed to OGA inhibition, rather than off-target effects.
7. Thiamet G (1 μ M): An OGA inhibitor structurally unrelated to GlcNAcstatin

2.2 Methods

1. Cell Culture: Cell culture flasks (Corning, Sigma Aldrich) were coated with 10ml of gelatine for 20 minutes at 37° C to ensure adherence to the flask. Gelatine was aspirated using a glass Pasteur pipette and 10ml of GMEM+LIF (for maintenance) containing approximately 1million cells from 46C cell line were added in the flask. After \pm 48h of cell culture, cells reached 80% confluence and the cells had to be re-plated in a new flask. Old media was aspirated using a glass Pasteur pipette and the cells were gently washed with 10ml of dPBS (1minute each wash). 1ml of Accutase was added directly to the cells and the flask was agitated until the cells came to a single cell suspension state, as evaluated by light microscopy. Media was then added to the cell suspension by gently pipetting up and down in order to remove any remaining cells stuck on to the flask surface. The suspension was then transferred in a 15ml Falcon tube and centrifuged at 800g for 5 minutes. After the end of centrifugation, the excess media was carefully aspirated and the pellet was re-suspended in 10ml of fresh media. The concentration of the cells in the suspension was calculated with a haemocytometer. The appropriate amount of cell suspension containing 1million cells was transferred into a new flask and media was added until the total volume was 10 ml (25cm² Flask) or 20ml (25cm² Flask).
2. Alkaline Phosphatase (AP) staining of cells with Sigma Kit
Protocol described in the kit provided.

3. Western Blotting: Described in chapter 2
4. Counting Protocol: To prepare the haemocytometer, the surface is carefully cleaned with ethanol and the coverslip is placed over the counting surface before adding the cell suspension. A small volume of cell containing media is pipetted onto a haemocytometer. Enough volume should be pipetted, in order to cover the whole surface. Cell suspensions should be dilute enough, so that the cells do not overlap each other on the grid. Also, before a sample is taken, the cell suspension is thoroughly mixed, so that the sample will be representative. The haemocytometer is then placed under the microscope. The grid contains 9 large squares. Each square has a surface of 1mm^2 and the depth of the chamber is 0.1mm . So each square represents a volume of 0.1mm^3 . 1ml is 1mm^3 and according to that we can calculate the total number of cells in the cell suspension.
5. Lactate Dehydrogenase (LDH) assay: LDH is a soluble cytosolic enzyme present in most eukaryotic cells. Toxic compounds lead to the release of LDH in the culture medium, due to damage of plasma membrane. Since LDH is a fairly stable enzyme, has been widely used to evaluate the presence of damage and toxicity of tissue and cells. The increase of the LDH in the concentration of a culture's supernatant is proportional to the number of lysed cells. The assay used here provides a photometric method to measure LDH activity. The reaction velocity is determined by measuring the decrease in absorbance resulting from the oxidation of NADH. $\text{NADH} + \text{pyruvate} \rightarrow \text{lactate} + \text{NAD}^+$. In each well $10\mu\text{L}$ of sample + $840\mu\text{L}$ Imidazole + $10\mu\text{L}$ DTT + $100\mu\text{L}$ NADH. The plate is carefully mixed. At last 50mL of pyruvate (substrate) are added in each well and the plate is placed into the photometer to measure the absorbance of the sample.

3 Results

3.1 Western GNS

To confirm that the known effects of GlcNAcstatin are applicable to ES cells, cells were plated in GMEM + serum + LIF + GNS, and at the time points stated in Figure 3-1. Samples were lysed and equalised for Western Blotting according to the described protocol.

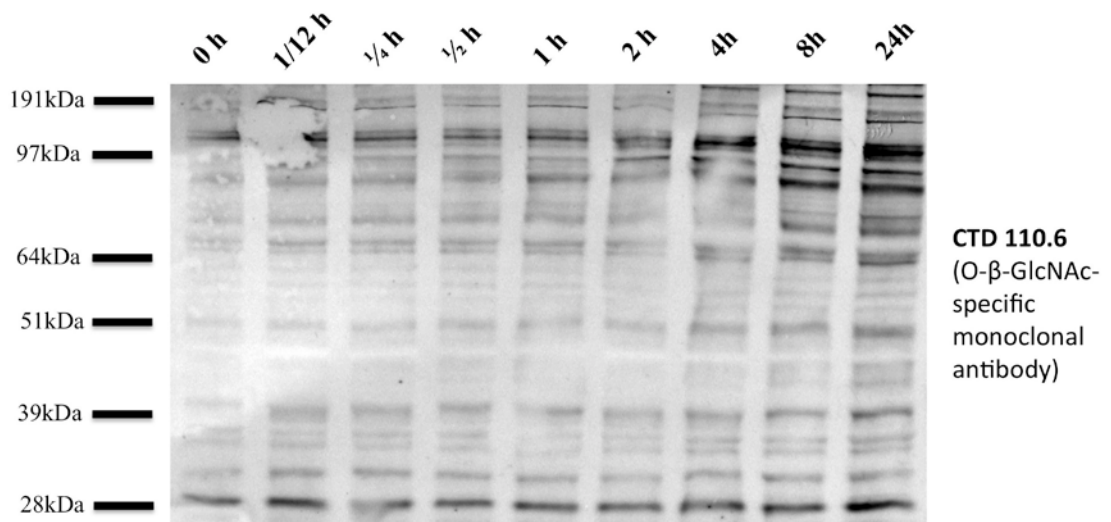


Figure 3-1: Western blot analysis for O-GlcNAc levels after adding GNS in the cell culture (Time points: 0min, 5min, 15min, 30min, 1h, 2h, 4h, 8h, 24h)

Figure 3-1 displays multiple bands (representing O-GlcNAcylated proteins) with increasing density as cells are incubated for longer periods of time with the inhibitor (GNS). GNS blocks OGA. OGA inhibition causes a marked increase in global O-GlcNAc levels.

3.2 Growth Curves

To examine the effects of OGA inhibition in the proliferating capacity of the ES cells, a seven-day time course experiment was performed. Every 24 hours the concentration and the number of total cells were calculated. As control, a biologically inactive isomer of GNS was used (GLS) along with a second O-GlcNAc inhibitor (Thiamet G).

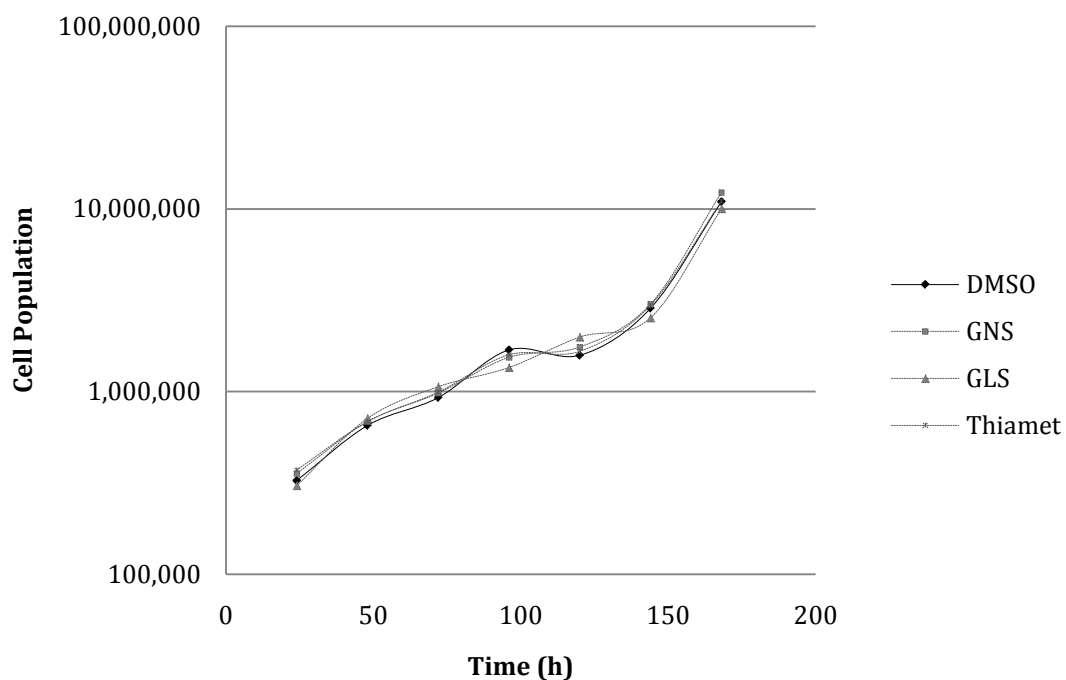


Figure 3-2: 7-Day time course experiment examining the effects of OGA inhibitors in cell proliferation.

Examination of the graph reveals no effects on cell proliferation even after prolonged treatment with GNS.

3.3 Cell viability

In order to estimate possible toxic effects of GNS after prolonged treatment with the cells, a LDH assay was used to measure the enzymatic activity of the entire population of cells. No significant differences were observed (Figure 3-3).

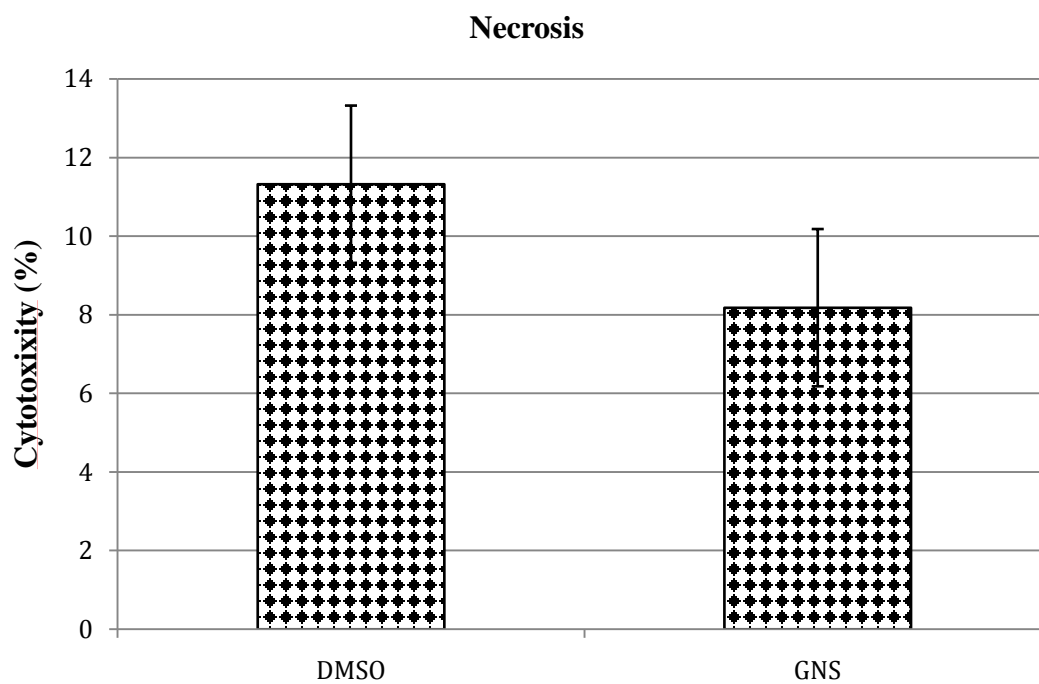


Figure 3-3: LDH assay in order to investigate possible toxic effects of GNS

3.4 Scores

Alkaline phosphatase is a phenotypic marker of pluripotent stem cells. While is expressed in most cell types, its expression is highly elevated in pluripotent stem cells. In the present experiment, we compare the effect of various media (media with or without GNS in the presence or absence of LIF) in the differentiating capacity of the cells.

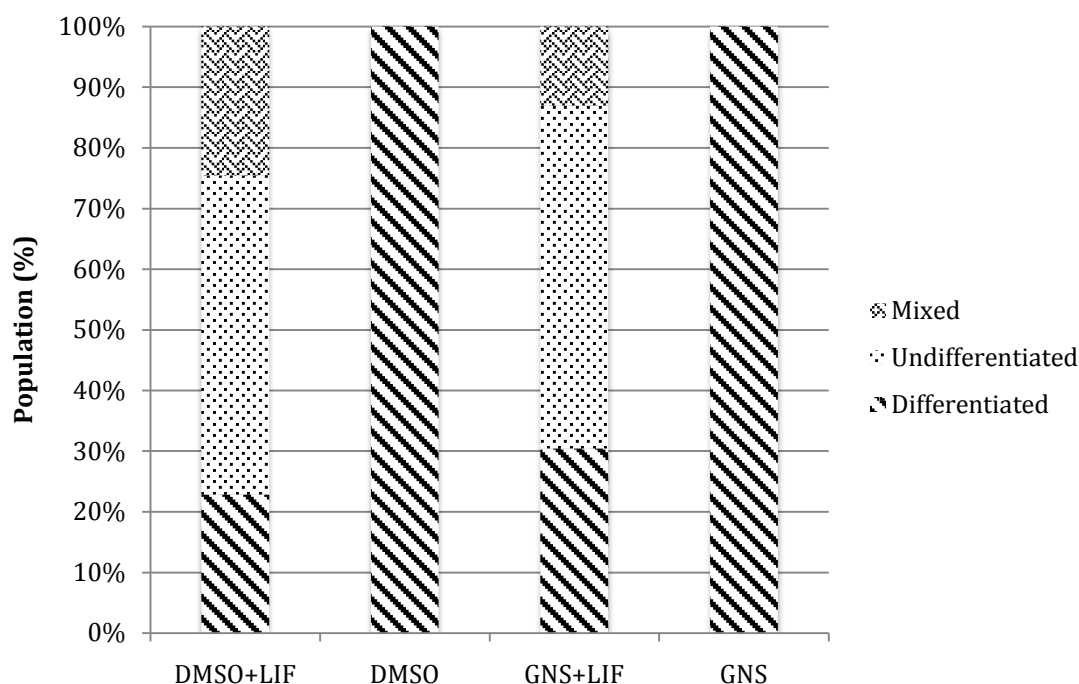


Figure 3-4: Cells cultures were treated with combination of three different compounds examining if inducing O-GlcNAcylation in presence or absence of LIF affects ES cell self-renewal.

Our results demonstrate that ES cell differentiation remains unaffected under conditions of increased O-GlcNAc signalling

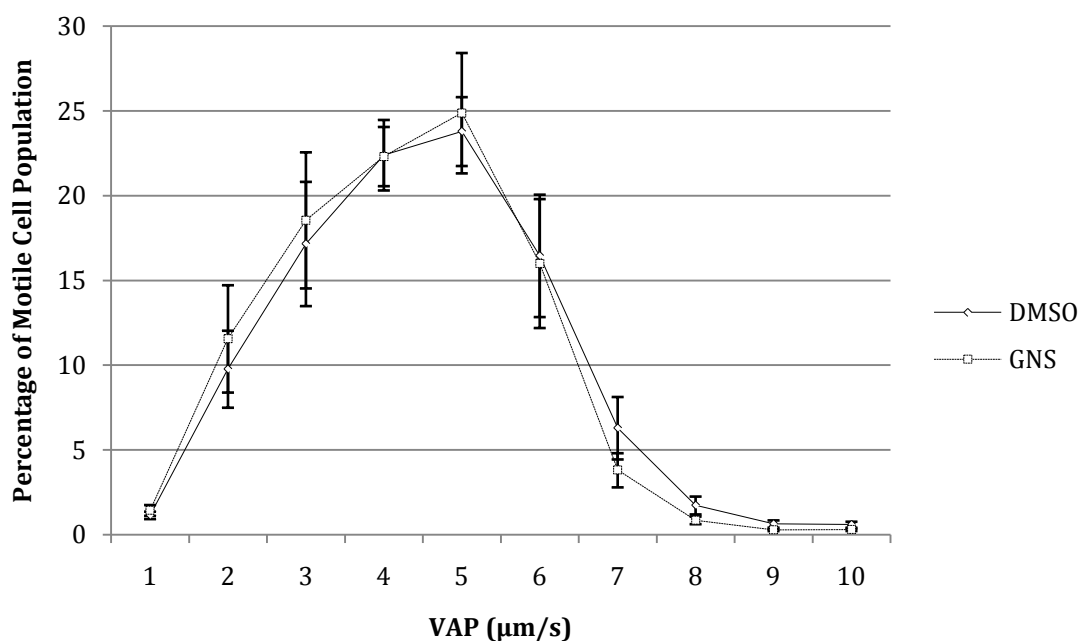
3 Discussion

In the final part of the project we examine the effects of the induction of O-GlcNAcylation in stem cells. Stem cells have the ability to self-renew and they have huge developmental capacity. Their differentiation is controlled by the interaction of multiple signalling pathways. These signalling pathways are typically mediated by post-translational modifications. O-GlcNAcylation is one such modification, which although regulates the function of several proteins, little is known about its effects in stem cells. Our experiments prove that induction of O-GlcNAcylation has no effects in cell viability or proliferation even after prolonged treatment. This comes in contrast with latest data suggesting, that O-GlcNAc signaling causes a differentiation deficiency in serum-free monolayer conditions. These data suggest that O-GlcNAc signaling stimulates Wnt transcriptional activity by up-regulating the transcriptional activator Lef1 leading to reduced entry of ES cells into a differentiated state.

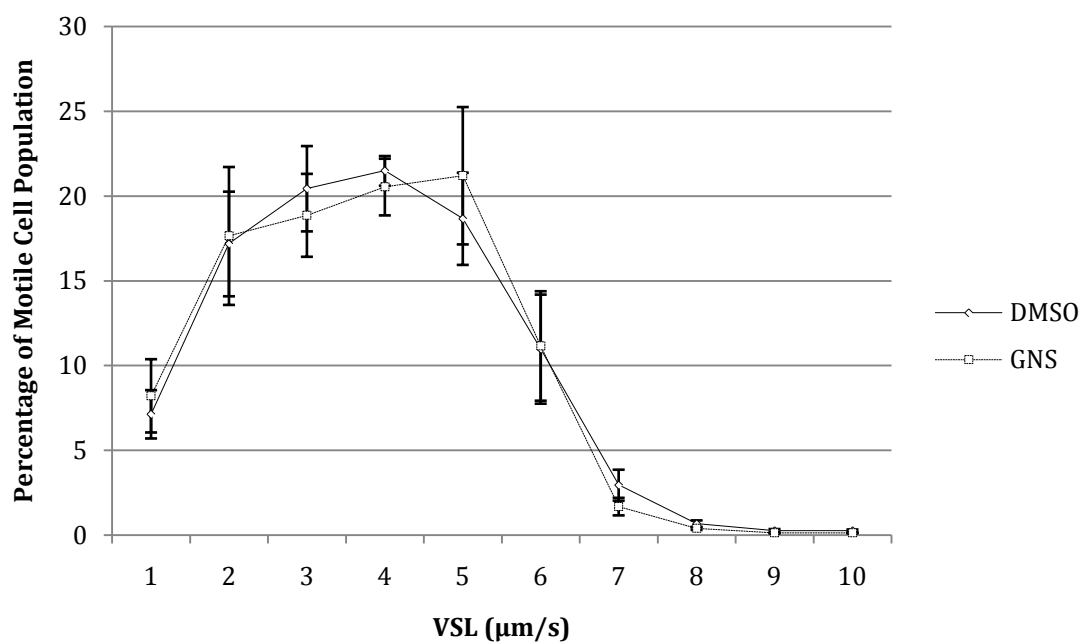
4 Additional motility data – frequency distributions

Motility frequency distributions, provide an arrangement of the values that each motility variable takes in a sample. So in addition to the information extracted from the average values presented in Chapter 4, frequency distribution can sometimes provide additional information. In the experiments conducted, no additional information could be extracted, as in most cases, no significant differences in the distribution of the population, were observed.

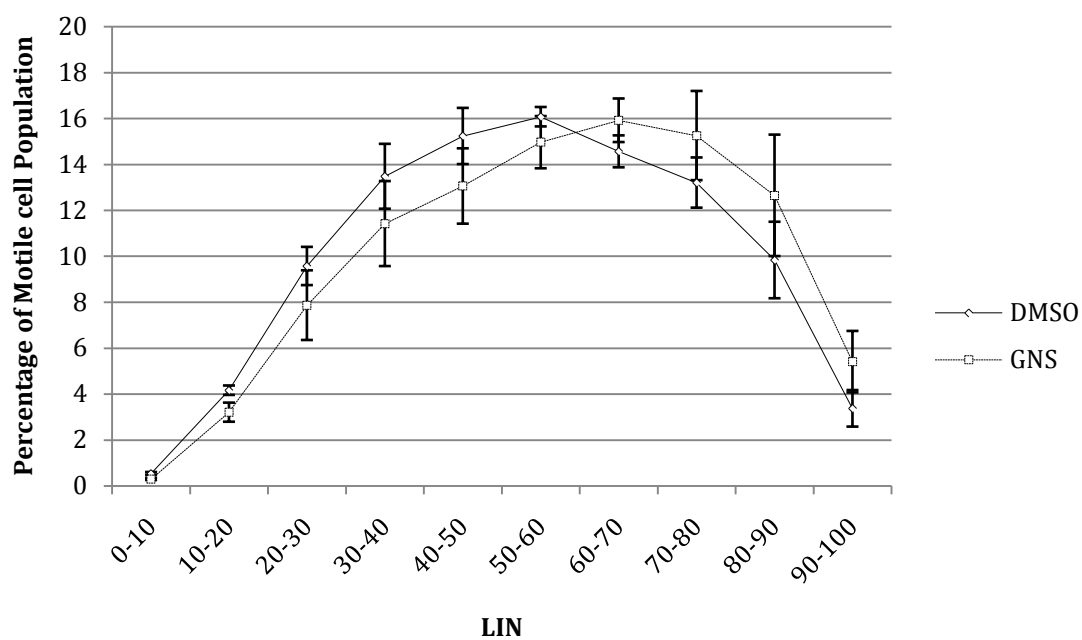
Inhibitor 1: GlcNAcstatin (GNS)



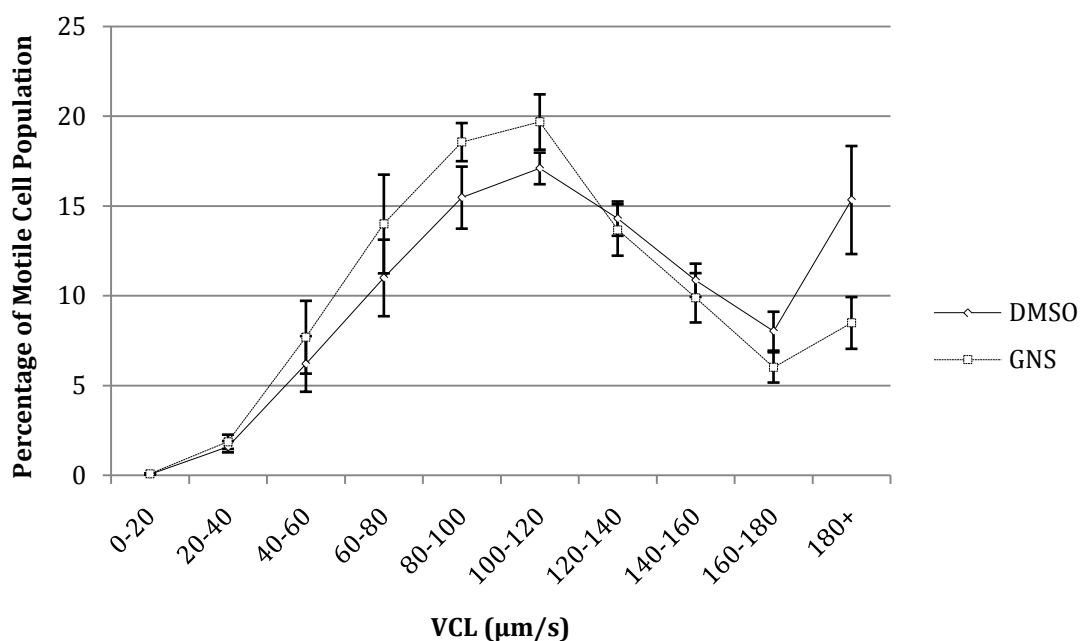
Influence of O-GlcNAcylation induction (GNS treatment) on human sperm path velocity. Values are mean \pm SEM for 10 individual donor ejaculates



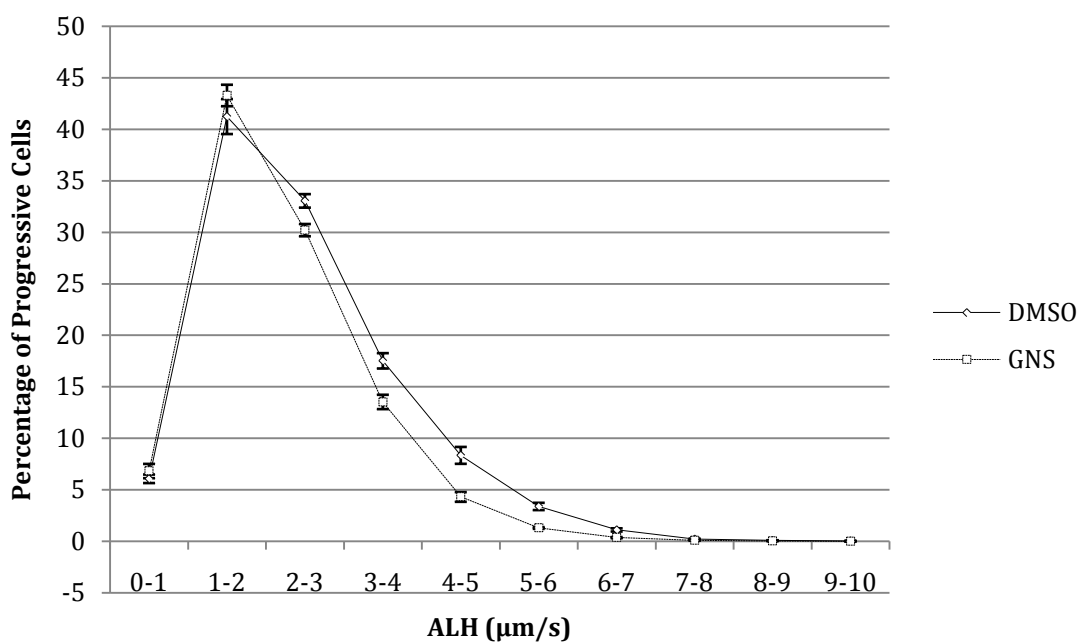
Influence of O-GlcNAcylation induction (GNS treatment) on human sperm progressive velocity. Values are mean \pm SEM for 10 individual donor ejaculates.



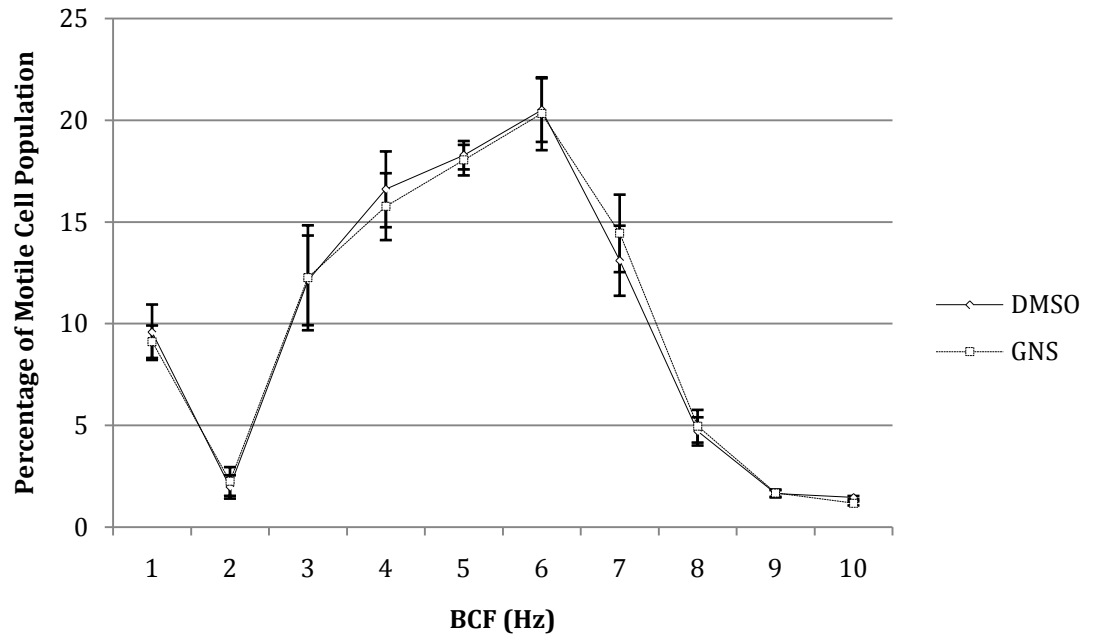
Influence of O-GlcNAcylation induction (GNS treatment) on human sperm linearity (LIN). LIN can be calculated from two individual velocity parameters, straight line velocity (VSL) and curvilinear velocity (VCL). Values are mean plus or minus SEM for 10 individual donors.



Influence of O-GlcNAcylation induction (GNS treatment) on human sperm Amplitude of lateral head displacement (ALH). Values are mean plus or minus SEM for 10 individual donor ejaculates.

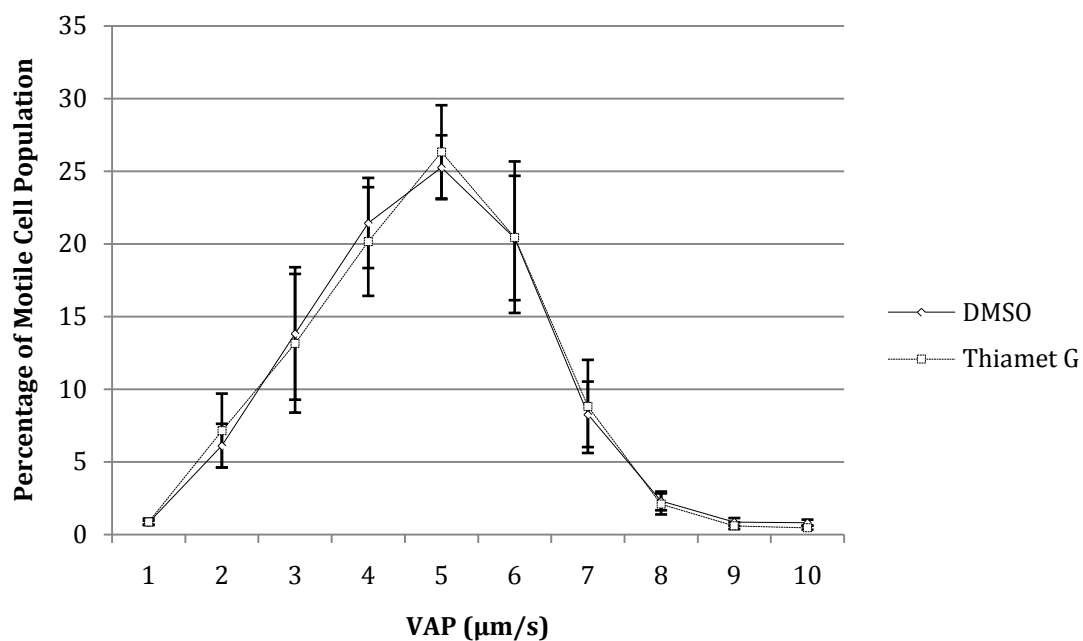


Influence of O-GlcNAcylation induction (GNS treatment) on human sperm curvilinear velocity (VCL). Values are mean \pm SEM for 10 individual donor ejaculates.



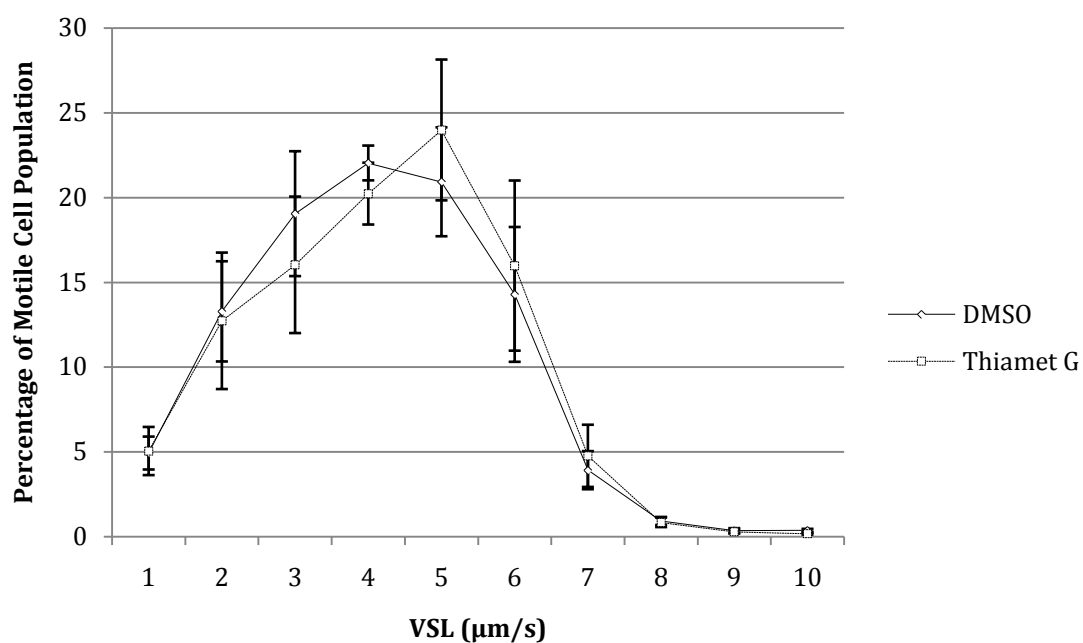
Influence of O-GlcNAcylation induction (GNS treatment) on Beat Cross Frequency.

Values are mean \pm SEM for 10 individual donor ejaculates.

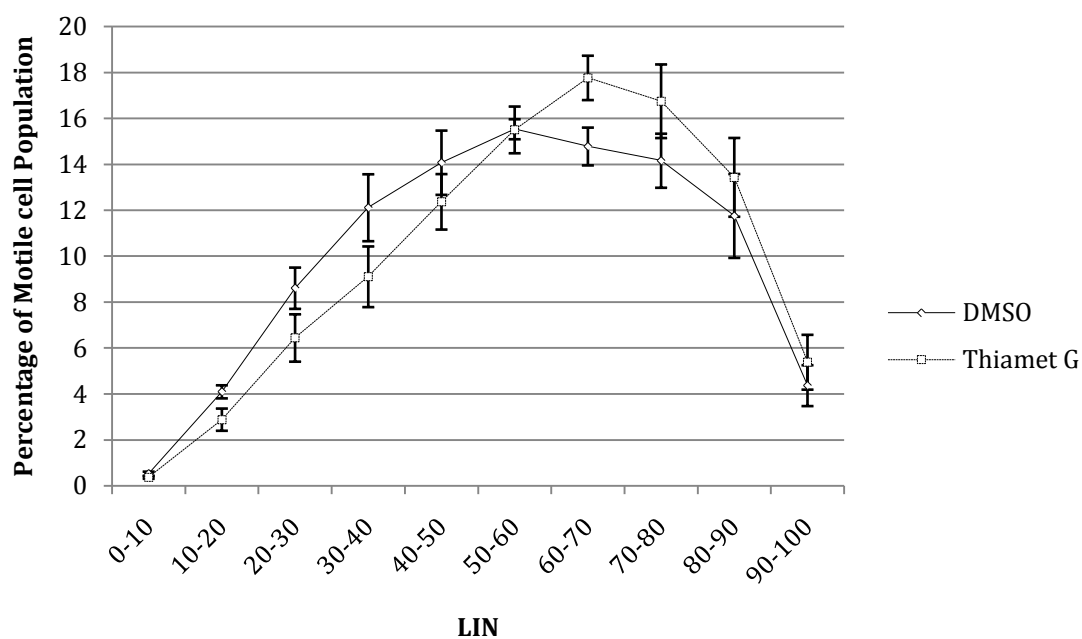
Inhibitor 2: Thiamet G

Influence of O-GlcNAcylation induction (Thiamet G) on human sperm path velocity.

Values are mean \pm SEM for 7 individual donor ejaculates.

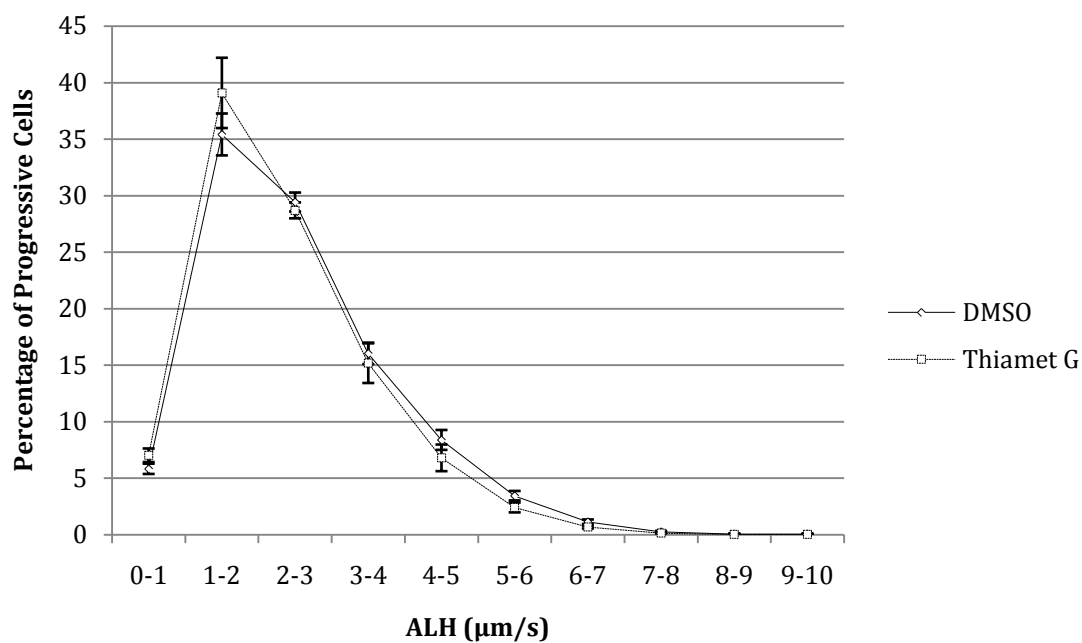


Influence of O-GlcNAcylation induction (Thiamet G) on human sperm progressive velocity. Values are mean \pm SEM for 7 individual donor ejaculates

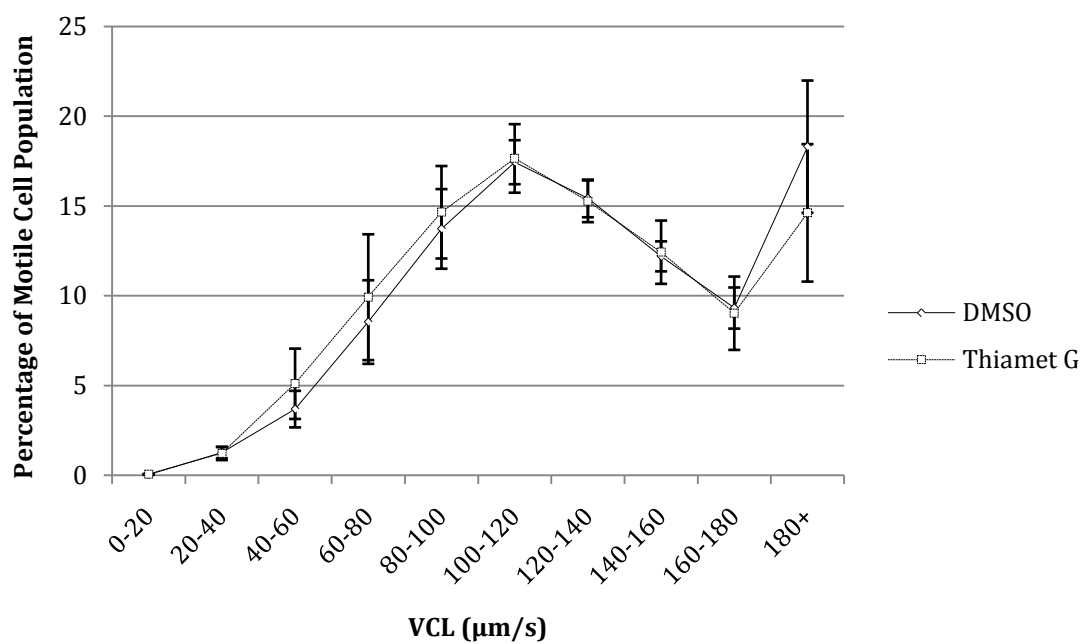


Influence of O-GlcNAcylation induction (Thiamet G) on human sperm linearity (LIN).

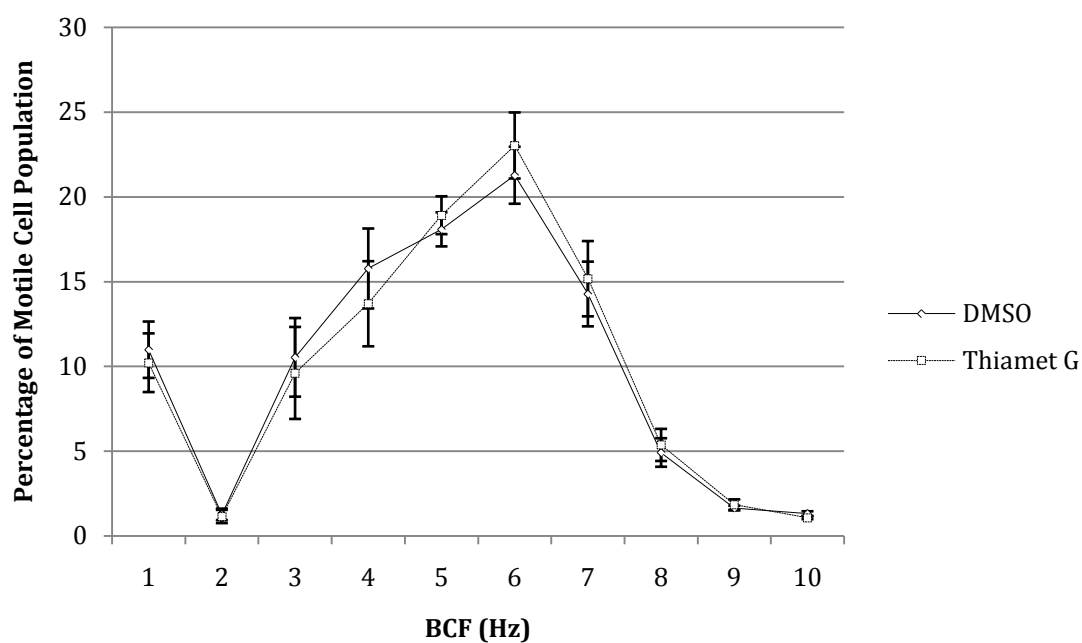
LIN can be calculated from two individual velocity parameters, straight line velocity (VSL) and curvilinear velocity (VCL). Values are mean plus or minus SEM for 7 individual donors.



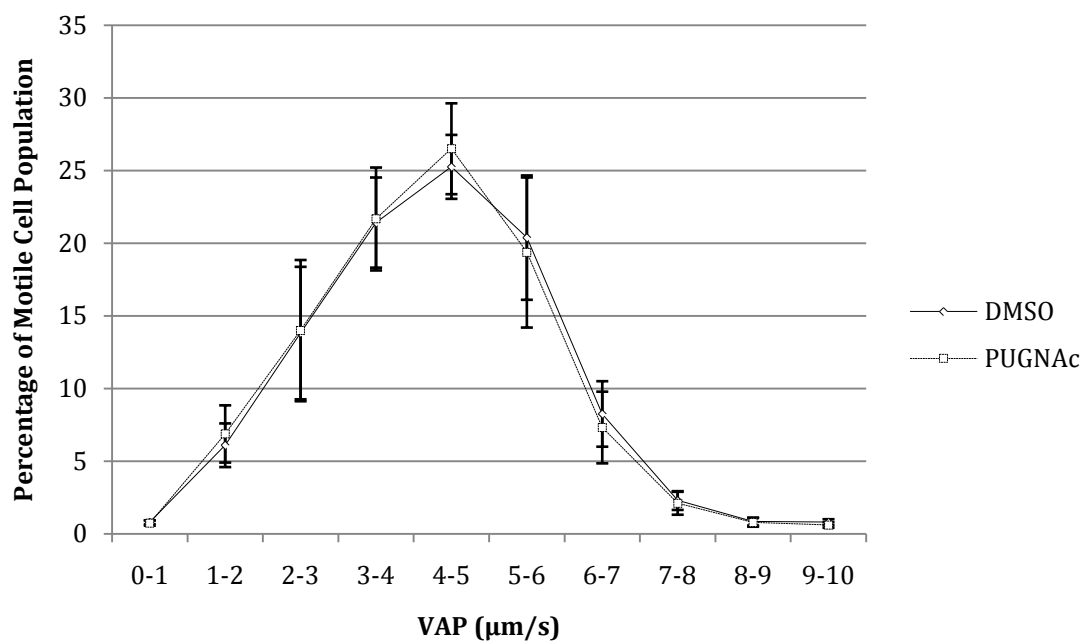
Influence of O-GlcNAcylation induction (Thiamet G) on human sperm Amplitude of lateral head displacement (ALH). Values are mean plus or minus SEM for 7 individual donor ejaculates.



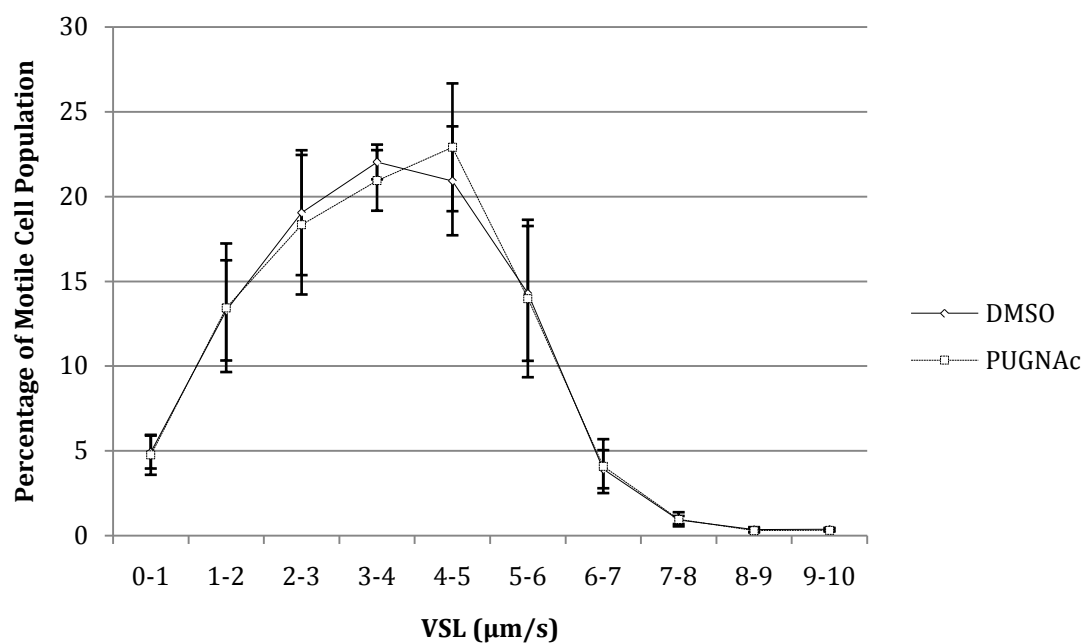
Influence of O-GlcNAcylation induction (Thiamet G) on human sperm curvilinear velocity (VCL). Values are mean \pm SEM for 7 individual donor ejaculates.



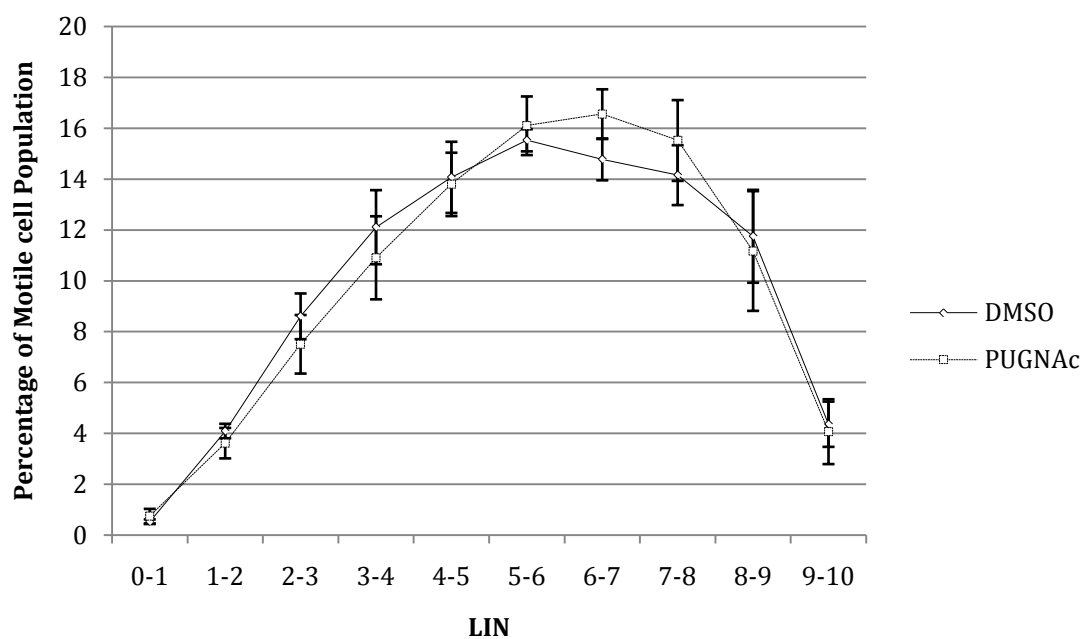
Influence of O-GlcNAcylation induction (Thiamet G) on Beat Cross Frequency. Values are mean \pm SEM for 7 individual donor ejaculates.

Inhibitor 3: PUGNAc

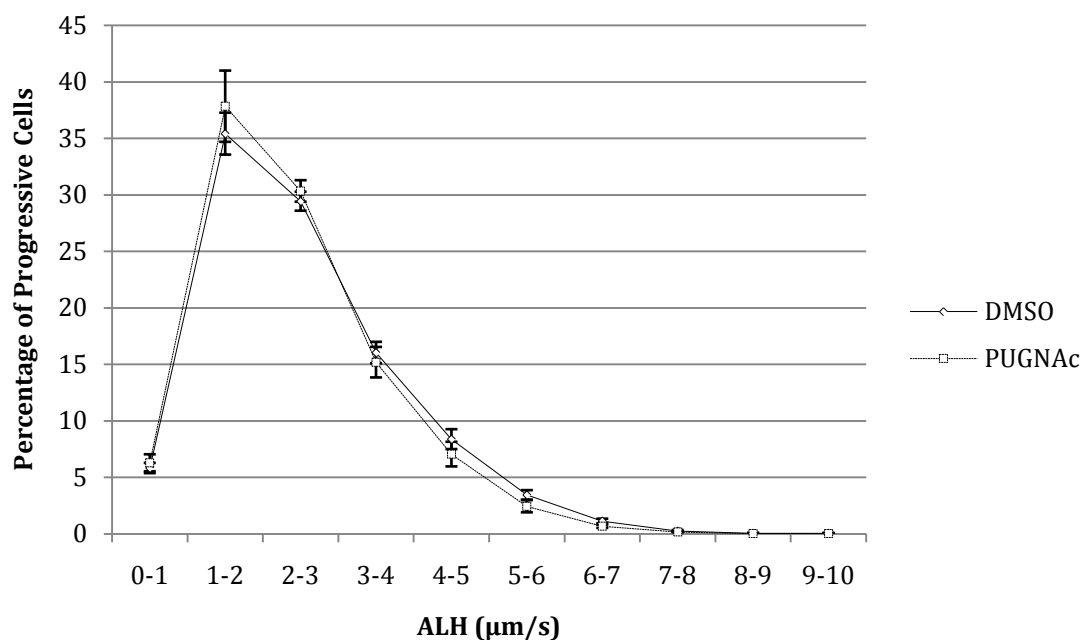
Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm path velocity. Values are mean \pm SEM for 7 individual donor ejaculates.



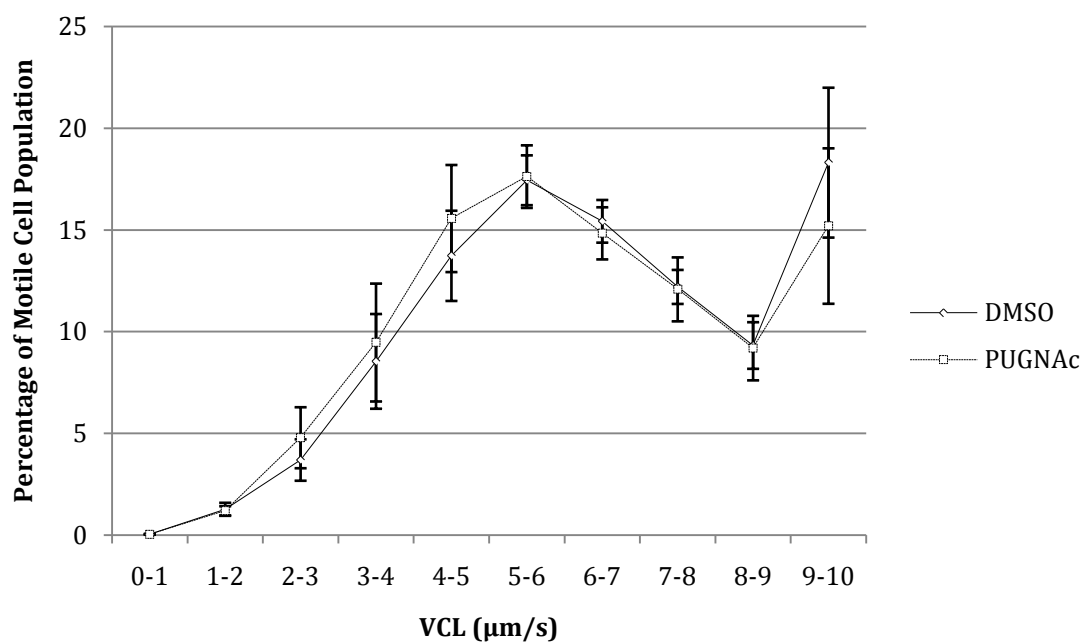
Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm progressive velocity. Values are mean \pm SEM for 7 individual donor ejaculates.



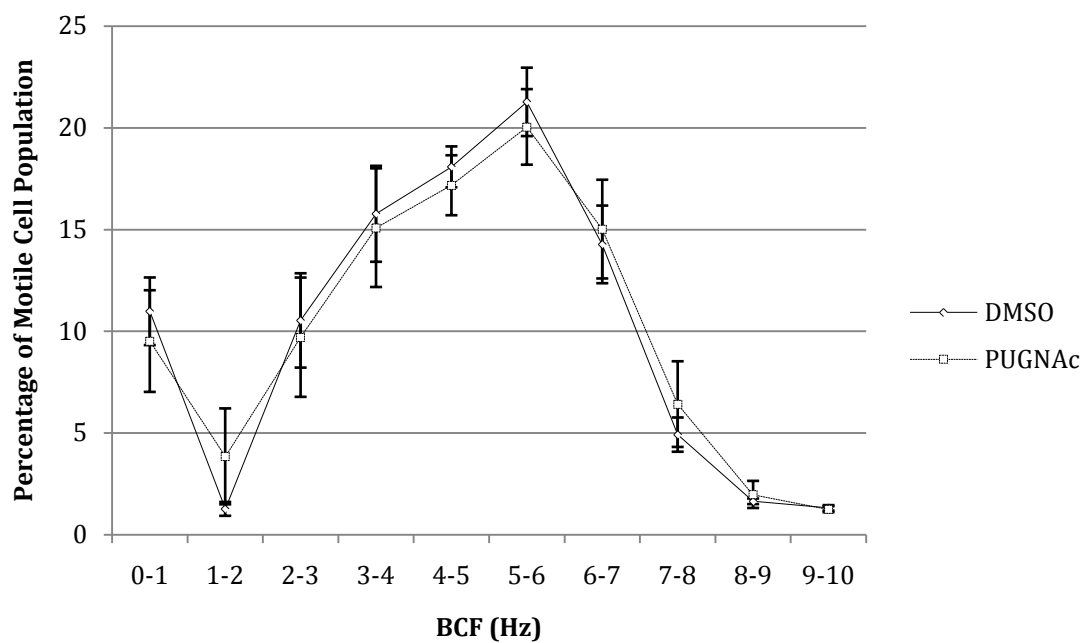
Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm linearity (LIN). LIN can be calculated from two individual velocity parameters, straight line velocity (VSL) and curvilinear velocity (VCL). Values are mean plus or minus SEM for 7 individual donors.



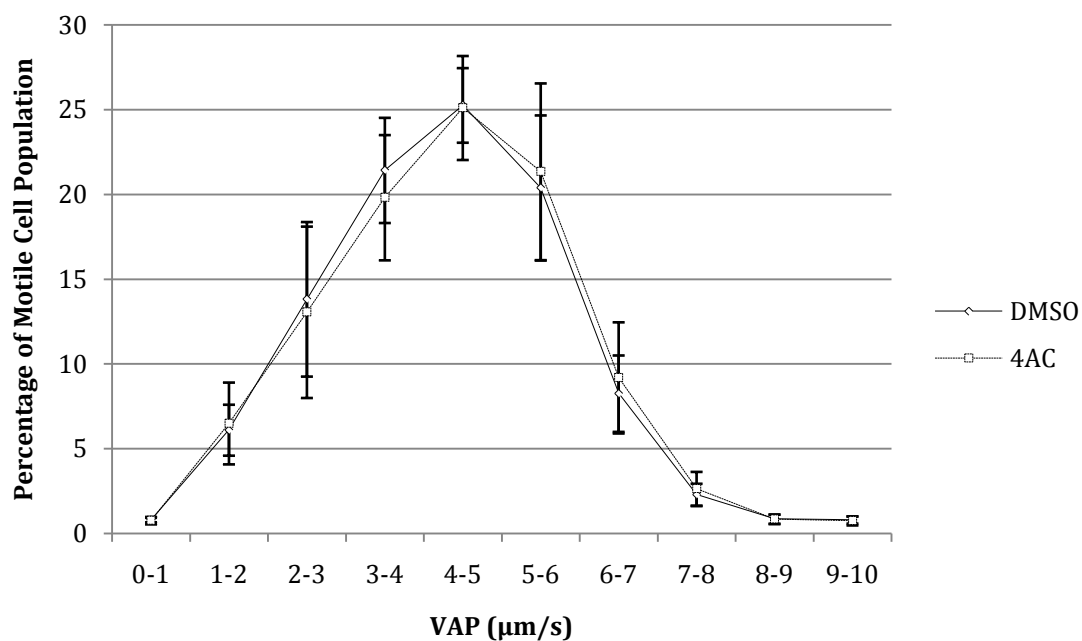
Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm Amplitude of lateral head displacement (ALH). Values are mean plus or minus SEM for 7 individual donor ejaculates.



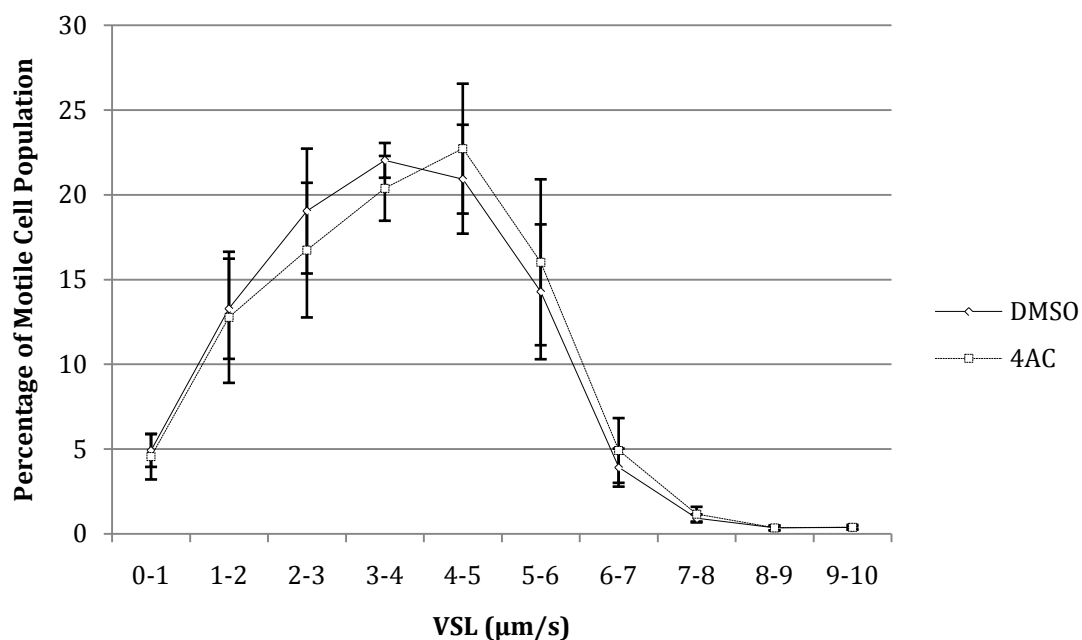
Influence of O-GlcNAcylation induction (PUGNAc treatment) on human sperm curvilinear velocity (VCL). Values are mean \pm SEM for 7 individual donor ejaculates.



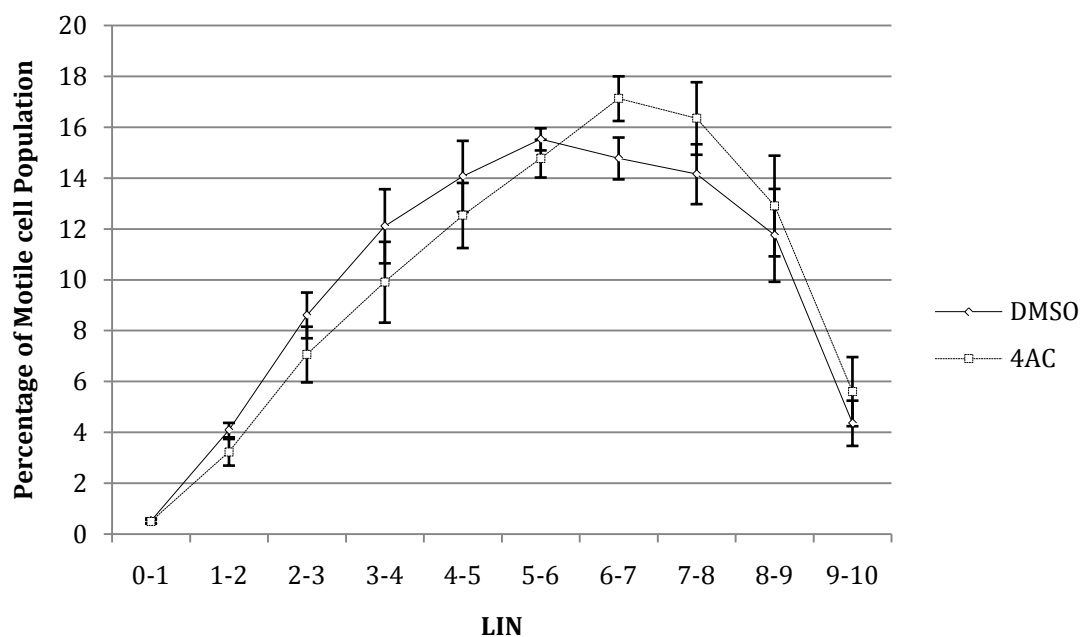
Influence of O-GlcNAcylation induction (PUGNAc treatment) on Beat Cross Frequency. Values are mean \pm SEM for 7 individual donor ejaculates.

Inhibitor 4: 4Ac5S-GlcNAc

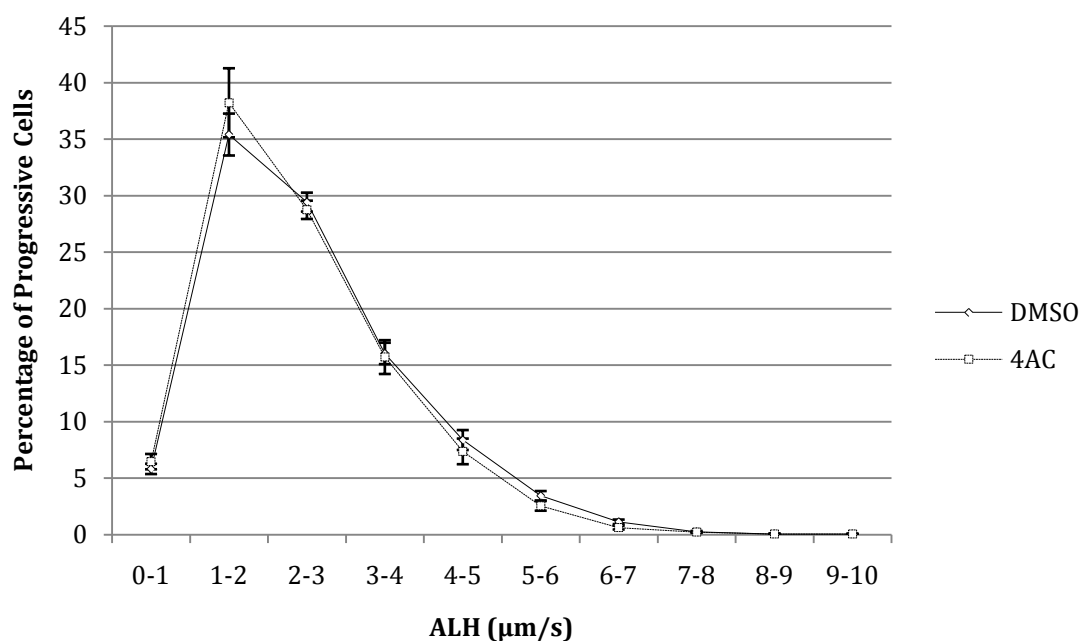
Influence of 4Ac5S-GlcNAc treatment (OGT inhibition) on human sperm path velocity. Values are mean \pm SEM for 7 individual donor ejaculates.



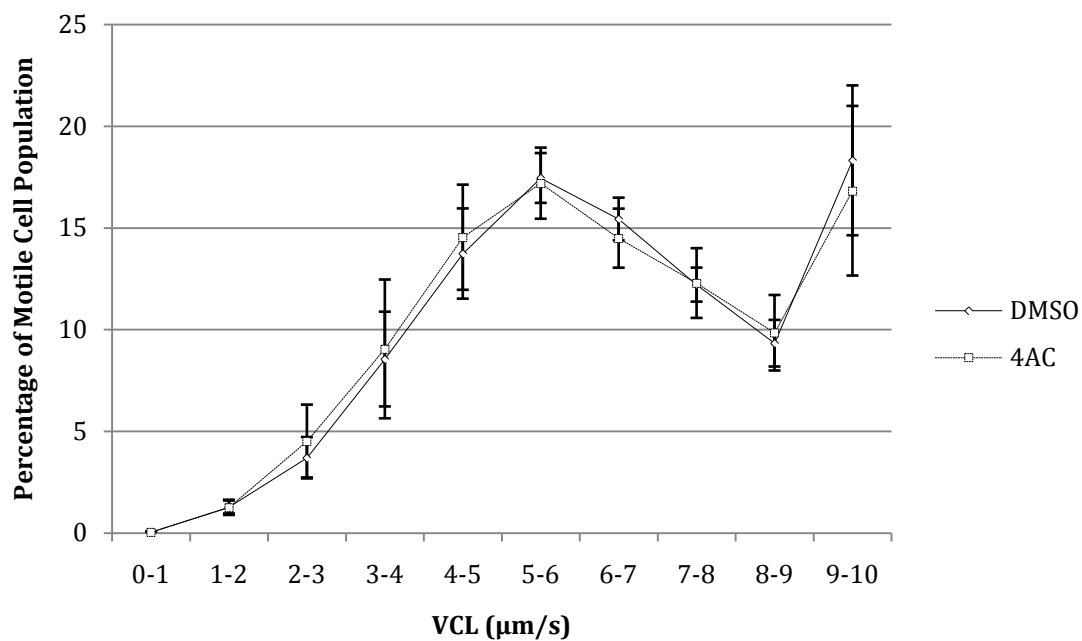
Influence of 4Ac5S-GlcNAc treatment (OGT inhibition) on human sperm progressive velocity. Values are mean \pm SEM for 7 individual donor ejaculates.



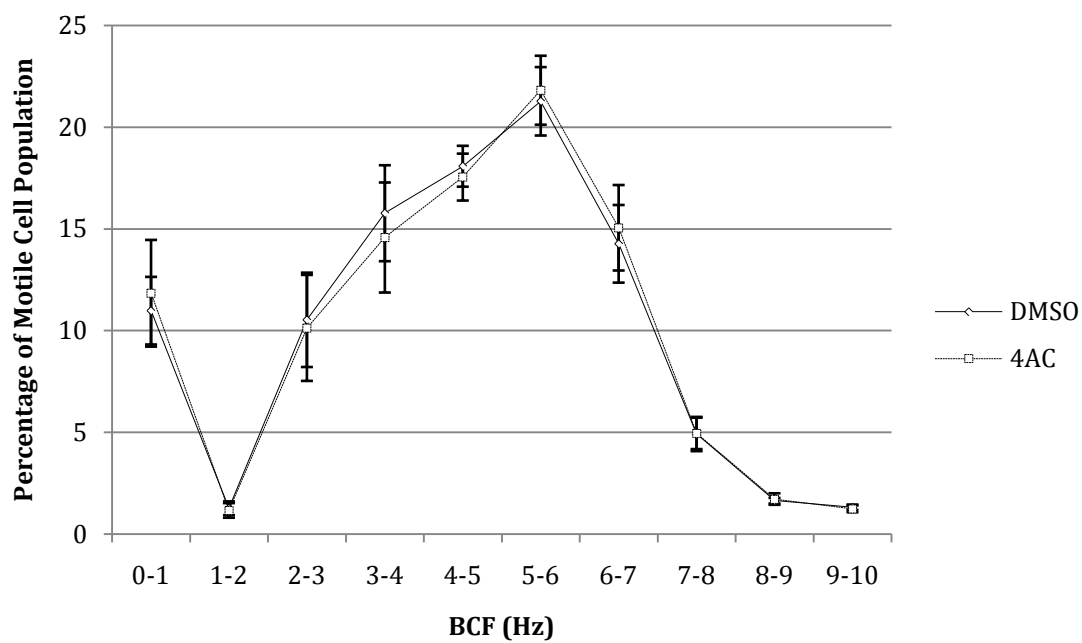
Influence of O-GlcNAcylation induction (4Ac5S-GlcNAc treatment) on human sperm linearity (LIN). LIN can be calculated from two individual velocity parameters, straight line velocity (VSL) and curvilinear velocity (VCL). Values are mean plus or minus SEM for 7 individual donors.



Influence of 4Ac5S-GlcNAc treatment (OGT inhibition) on human sperm Amplitude of lateral head displacement (ALH). Values are mean plus or minus SEM for 7 individual donor ejaculates.



Influence of 4Ac5S-GlcNAc treatment (OGT inhibition) on human sperm curvilinear velocity (VCL). Values are mean \pm SEM for 7 individual donor ejaculates.



Influence of 4Ac5S-GlcNAc treatment (OGT inhibition) on Beat Cross Frequency. Values are mean \pm SEM for 7 individual donor ejaculates

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